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16. ABSTRACT

The Cholinesterase Antagonist Monitors ("CAM's") normally use cholinesterase as the sensor in the detection of organophosphate and carbamate pesticides. The present investigation has been concerned with a search for alternate enzymes that could be used in the CAM system and that would enable it to detect a variety of other types of environmentally important toxic chemicals including chlorinated hydrocarbons, phenols, aryl phosphates, cyanide, heavy metals, etc. Five enzymes including alkaline phosphatase, carboxyl esterase, glucose oxidase, carbonic anhydrase, and hexokinase have been incubated with dilute solutions of toxic chemicals and the degree of enzyme inhibition measured. Two of the enzymes, hexokinase and carbonic anhydrase, were inhibited by low levels of toxic test compounds. It is concluded that these and similar enzymes are of potential value in the detection and monitoring of toxic substances in water.

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Alternate Enzymes for Use in Cholinesterase Antagonist Monitors ("CAM's")

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May 1980

ALTERNATE ENZYMES FOR USE IN CHOLINESTERASE ANTAGONIST MONITORS ("CAM's")

by

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FOREWORD

When energy and material resources are extracted, processed, converted, and used, the related pollutional impacts on our environment and even on our health often require that new and increasingly efficient pollution control methods be used. The Industrial Environmental Research Laboratory-Cincinnati (IERL-Ci) assists in developing and demonstrating new and improved methodologies that will meet these needs both efficiently and economically.

This report describes a search for alternate enzymes to be used in the Cholinesterase Antagonist Monitor (CAM) systems. When operated with cholinesterase, the CAM instruments work well for the detection of organophosphate and carbamate pesticides on a real-time basis, but they cannot detect compounds that do not inhibit cholinesterase. In the present investigation, a group of commercially available enzymes was investigated to determine potential usefulness in the CAM instruments for the detection of non-cholinesterase-inhibiting, toxic materials in water, such as chlorinated hydrocarbons, aryl phosphates, phenols, and heavy metals. Thus, CAM systems, designed for monitoring of water supplies and municipal and industrial effluents, can be used to alert responsible personnel to a spill of a wide range of toxic and hazardous materials and can be used as a means of tracing the course of these materials in waterways. Information on this subject beyond that supplied here may be obtained by contacting the Oil and Hazardous Materials Spills Branch, IERL-Ci, USEPA, Edison, New Jersey 08817.

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ABSTRACT

The CAM series of instruments ("CAM" is an acronym for "cholinesterase antagonist monitor") was previously constructed to automatically monitor the levels of organophosphate and carbamate pesticides in water. In these real-time monitoring devices an immobilized enzyme, cholinesterase, is inactivated in the presence of organophosphate and carbamate pesticides. The residual activity of the enzyme, related to the level of inhibitor, is determined by measuring the electrochemical response of the system to a substrate readily hydrolyzed by the enzyme.

It was expected that the CAM system could be used to detect hazardous materials other than organophosphate and carbamate pesticides if enzymes that were inhibited by other toxic and hazardous materials of interest could be found. Therefore, a preliminary study was conducted to determine how the CAM system could be adapted to respond to toxic materials, such as chlorinated hydrocarbons, phenols, aryl phosphates, cyanides, and heavy metals in water. The approach consisted of a search for enzymes that: (1) could be used in the CAM systems in place of immobilized cholinesterase, (2) would be inhibited by low levels of one or more of the toxic substances of interest, and (3) were detectable by electrochemical means. In this search, more than 20 enzymes were evaluated to see whether they met the following requirements: (1) were commercially available at moderate cost, (2) were sufficiently stable for study, (3) required no expensive cofactors, and (4) used substrates that were inexpensive and changed redox potentials as a result of enzyme action.

Laboratory investigations of the following five enzymes were made: alkaline phosphatase (*E. coli*), carboxylesterase (hog liver), glucose oxidase (*Aspergillus niger*), carbonic anhydrase (bovine erythrocyte), and hexokinase (yeast). Inhibition studies of these enzymes showed that glucose oxidase, carboxylesterase, and alkaline phosphatase were not significantly inhibited (>15%) by 10^{-4} M solutions of the toxic test materials. Carbonic anhydrase was significantly inhibited by 10^{-4} M solutions of methoxychlor, DDT, toxaphene, pentachlorophenol, cyanide ion, and cadmium and mercury compounds. Hexokinase was significantly inhibited by 10^{-4} M or 10^{-5} M solutions of chlordane, DDT, heptachlor, aldrin, lindane, methoxychlor, toxaphene, mercuric ion, and tri-o-cresyl phosphate.

Hexokinase and carbonic anhydrase are potentially useful in a CAM-type instrument for detection of selected hazardous materials. However, neither enzyme has yet been adapted to the CAM system.

This report was submitted in fulfillment of Contract No. 68-03-0299, Task No. 3, by Midwest Research Institute under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period 3 November 1976 to 3 July 1977 and work was completed as of 18 November 1977.

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ABBREVIATIONS

ADP --Adenosine diphosphate

ATP --Adenosine triphosphate

BCA --Bovine carbonic anhydrase

CAM --Cholinesterase Antagonist Monitor, general term for those instruments using cholinesterase in an electrochemical cell for detection and monitoring of cholinesterase antagonists

CAM-1 --Cholinesterase Antagonist Monitor, automatically detects and monitors water supplies for cholinesterase inhibitors

CAM-4 --Battery-operated, portable version of CAM-1

DDD --2,2-Bis(p-chlorophenyl)-1,1-dichloroethane

DDT --2,2-Bis(p-chlorophenyl)-1,1,1-trichloroethane

GTP --Guanosine triphosphate

NAD --Oxidized form of the co-factor, nicotinamide adenine dinucleotide (also called diphosphopyridine nucleotide)

NADH --Reduced form of NAD (see above)

p-NPA --p-Nitrophenyl acetate

p-NPTA --p-Nitrophenyl thioacetate

p-NTP --p-Nitrothiophenol

2,4,5-T --2,4,5-Trichlorophenoxyacetic acid

TOCP --Tri-o-cresyl phosphate

Tris --Tris(hydroxymethyl)-aminomethane partially neutralized with HCl; a buffer

TTP --Thymidine triphosphate

UTP --Uridine triphosphate

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The work upon which this publication is based was performed pursuant to the earlier Contract No. 68-01-0038 and the current Contract No. 68-03-0299 with the Environmental Protection Agency; this report describes the work on Task III of the current contract. Task I of this contract evaluated the response of the Cholinesterase Antagonist Monitor Model No. 1 (CAM-1) to a series of commercially available pesticides, including both organophosphates and carbamates. Task II covered the design, fabrication, and evaluation of a portable version of CAM-1, which has been designated as CAM-4. Task III was devoted to an investigation of alternate enzyme systems for use in CAM-1 and CAM-4. Work on all tasks has now been completed.

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SECTION 1

INTRODUCTION

As the manufacture and use of industrial and agricultural chemicals has accelerated, the danger of pollution of natural waterways has increased. This contract was performed pursuant to the Environmental Protection Agency's interest in the development of instrumental methods for the detection and continuous monitoring of hazardous materials spills.

The Cholinesterase Antagonist Monitor (CAM-1) was developed by Midwest Research Institute on an earlier EPA Contract (No. 68-01-0038). The instrument is capable of monitoring water for the presence of organophosphate and carbamate pesticides on a real-time basis. Detailed information regarding the theory of operation and the physical components of CAM-1 can be found in EPA Report No. R2-72-010, August 1972.

Task 1 of the present contract, entitled "Evaluation of a Warning Device for Organophosphate Hazardous Material Spills," delineated the sensitivity of the CAM-1 instrument to various pesticides. During Task II, entitled "CAM-4, A Portable Warning Device for Organophosphate Hazardous Material Spills," a portable version of the CAM--designated CAM-4--was developed and tested. The practical worth of the CAM system was thereby extended to include in-the-field monitoring.

This publication is a report on Task III, "Alternate Enzymes for Use in CAM Instruments." Presently, the detection capability of the CAM instruments is limited to toxic materials that inhibit the enzyme cholinesterase. The objective of Task III was to examine other enzyme systems for potential use in CAM to detect additional toxic materials.

SECTION 2

CONCLUSIONS

The present report is concerned with the investigation of a number of enzyme systems to determine their potential usefulness in the CAM instrument for the detection and monitoring of toxic substances in the aquatic environment. In enzyme-inhibition studies, measurements were made of the decrease in enzyme activity resulting from incubating candidate enzymes with dilute solutions of chlorinated hydrocarbons, phenols, aryl phosphates, cyanide ion, and heavy metal ions. As a result of these studies, the following conclusions can be drawn:

1. Carbonic anhydrase is inhibited by 5×10^{-5} M solutions of several chlorinated hydrocarbons and inorganic substances, such as pentachlorophenol, cyanide ion, and mercuric ion. Although carbonic anhydrase has not yet been adapted to the CAM system, such adaptation may be both practical and useful.

2. Hexokinase is inhibited by low levels (10^{-4} to 10^{-5} M) of DDT, chlordane, lindane, and toxaphene. It is also inhibited by mercuric ion and by tri-o-cresyl phosphate. Therefore, hexokinase is considered to be a potential sensor for these toxic substances and should be considered for adaptation to a real-time monitoring system.

3. Glucose oxidase, carboxylesterase, and alkaline phosphatase are not promising for use in CAM as sensors for the battery of toxic substances tested.

4. Although the aim of this study was to adapt new enzymes for use in the electrochemical detection system of the CAM instrument, immobilized horse serum cholinesterase and eel cholinesterase remain--at this time--the only enzymes that have been used successfully.

SECTION 3

RECOMMENDATIONS

On the basis of the preliminary investigation of "Alternate Enzymes for Use in Cholinesterase Antagonist Monitors," it is clear that little is known about the possible application of hundreds of enzymes to problems in the detection and monitoring of toxic and hazardous materials. However, this field appears promising. Therefore, the following recommendations are germane:

1. Studies on the potential use of carbonic anhydrase and hexokinase for the detection and monitoring of toxic substances--especially chlorinated hydrocarbons--in water should be continued.

2. A larger number of available enzymes should be screened to determine their potential sensitivity to toxic substances of interest, including chlorinated hydrocarbon pesticides, phenols, solvents, aryl phosphates such as tri-o-cresyl phosphate, and heavy metals.

3. Studies of the inhibition of oxidative enzymes should include the co-immobilization of these enzymes with the NAD-NADH system to demonstrate the practicality of using oxidative enzymes for the detection of toxic materials in water or air. These enzymes are especially important in the metabolism of living systems and should be particularly sensitive to highly toxic substances.

4. One or more promising enzymes should be selected and adapted for use in a CAM-like instrument for monitoring water or air on a real-time basis.

5. New breadboard enzyme-monitoring systems should be evaluated to determine their sensitivity to toxic materials of interest and their susceptibility to interferences in the environments in which they would be used.

SECTION 4

EXPERIMENTAL APPROACH

The framework of Task III permitted the examination of any of the 200 to 300 commercially available enzymes for detection of hazardous materials. As this was such a broad area for investigation, it was immediately apparent that arbitrary but logical restrictions on the selection of enzymes and toxic and hazardous materials for laboratory study were needed. Criteria for candidate enzymes and toxic materials of interest were outlined early in the program in order to ensure acquisition of the most useful information during the limited time available. A scheme for laboratory examination of candidate monitoring enzymes evolved. The following paragraphs describe the selection of toxic materials and candidate enzymes and the scheme for laboratory testing.

TOXIC MATERIALS OF INTEREST IN WATER MONITORING

It is recognized that virtually every substance has the potential for presenting some hazard to the public health or welfare depending upon its physical properties, its toxicity, and the quantity, time, and place of its discharge. As only a finite number of hazardous compounds could be tested in vitro with each enzyme in the laboratory, it was necessary to carefully determine which toxic substances were of greatest environmental concern and therefore could be considered as target compounds for extending the detection capabilities of the CAM instrument.[1,2,3] A review of EPA criteria for designation of hazardous substances was made.

EPA regulations (40 CFR 116) place designated hazardous substances into five categories (X,A,B,C,D) on the basis of reportable quantities (1,10,100, 1000,5000 lb, respectively). The determination of minimum reportable quantities is based on the chemical and toxicological properties of the substance of concern. Many of these hazardous substances can be placed in six chemical classes of interest to the present study. Materials in two classes, organophosphates and carbamates, are detectable by the CAM systems when operated with cholinesterase. Therefore, materials for this study were largely selected from four other classes: (1) chlorinated hydrocarbons, (2) cadmium compounds, (3) cyanide compounds, and (4) mercury compounds. In addition, several other hazardous materials were also examined. Table 1 lists materials tested and, where applicable, identifies the appropriate EPA "reportable quantity (RQ)" hazardous category.

TABLE 1. HAZARDOUS MATERIALS SELECTED FOR STUDY

Material	EPA Category	Material	EPA Category
Chlorinated hydrocarbons:		Mercury compounds:	
Aldrin	X	Mercuric acetate	—
Chlordane	X	Mercuric chloride	—
DDD	X		
DDT	X	Aryl phosphates:	
Endosulfan	X		
Endrin	X	Phenylphosphate, disodium salt	—
Heptachlor	X		
Lindane	X	Tri-o-cresyl phosphate	—
Methoxychlor	X	Tricresylphosphate (mixed cresols)	—
Pentachlorophenol	A		
Toxaphene	X		
Trichlorophenol	X	Cadmium compounds:	
2,4,5-T acids	B		
2,4,5-T esters	B	Cadmium chloride	B
Cyanide compounds:		Others:	
Potassium cyanide	A	Lead acetate	D
Sodium cyanide	A	Potassium permanganate	B
		Strontium acetate	—

SELECTION OF CANDIDATE ENZYMES

Prior to beginning laboratory work it was necessary to decide which enzymes showed greatest promise for detection of the target compounds and for adaptation to CAM. Twenty enzymes were considered for laboratory study because: (1) the literature indicated that they were inhibited by the toxic substances of interest and (2) the enzymes yield sulfhydryl groups upon reaction with substrates, making them more easily adaptable to the CAM system (the utility of cholinesterase in the CAM system depends on the enzymatic production of sulfhydryl groups). The following 20 enzymes by no means include all potentially useful monitoring enzymes but provided the starting point for this work:

Glucose oxidase (1.1.3.4)*	Carboxylesterase (3.1.1.1)
Xanthine oxidase (1.2.3.2)	Arylesterase (3.1.1.2)
Pyruvate dehydrogenase (1.2.4.1)	Lipase (3.1.1.3)
Succinic dehydrogenase (1.3.99.1)	Acetylesterase (3.1.1.6)
Glutathione reductase (1.6.4.2)	Alkaline phosphatase (3.1.3.1)
Thiol oxidase (1.8.3.2)	Acid phosphatase (3.1.3.2)
Glutathione dehydrogenase (1.8.5.1)	Urease (3.5.1.5)
Cytochrome oxidase (1.9.3.1)	ATPase (3.6.1.3)
Catalase (1.11.1.6)	Carbonic anhydrase (4.2.1.1)
Hexokinase (2.7.1.1)	S-Alkyl cysteine lyase (4.4.1.6)

Information pertinent to the selection of enzymes for laboratory testing was assembled for each of the 20 candidate enzymes in the form of fact sheets. Tne fact sheets for all candidate enzymes are included in this report as Appendix A.

The relative merits of the various enzymes, based on the following enzyme selection criteria, were considered by a panel of five MRI biochemists:

1. Commercial availability and cost: Use of enzymes not commercially available is precluded. Although cost may be minimized by enzyme immobilization, which permits reuse, it should not be so high as to prohibit eventual routine use of the immobilized enzyme in a CAM-like water monitor.

2. Stability: Extremely labile enzymes are difficult to handle and have a low likelihood of succesful use, therefore, enzymes for laboratory study should be reasonably stable.

3. Specific activity: Specific activity is an expression of the catalytic efficiency of the enzyme. Enzymes with high specific activity are desired since they are expected to have the greatest bioamplification factor and to be the most sensitive to pollutants.

4. Substrate specificity: Enzymes with low substrate specificity are versatile and should be easily adaptable to the CAM instruments.

5. Cofactor requirements: A requirement by an enzyme for cofactors, such as NAD-NADH, imposes an additional burden. Regeneration or reuse of expensive cofactors presents complications that cannot be satisfactorily studied within the scope of the current contract. Therefore, enzymes not requiring cofactors are preferred.

6. Response to inhibitors: The candidate enzyme should be inhibited by low concentrations of the hazardous materials of interest in water monitoring. Literature reports of inhibition are considered, when available.

*Enzyme Commission numbers for enzyme identification.

7. Electrochemical detection: The enzyme should produce or destroy a substance that is easily oxidized or reduced electrochemically. Indirectly, the enzyme may produce a product that, through reaction with another material, becomes either easily oxidizable or reducible.

A point system for judging the relative merits of candidate enzymes was devised. In each of the following areas, a maximum of three points was awarded: (1) reported inhibition by pollutants of interest, (2) expected adaptability to the CAM instrument, and (3) other factors such as cost and cofactor requirements (1-5 above).

As a result of discussions and a review of the fact sheets, the five panel members mutually agreed on the following point assignments:

Enzyme	Points
Carbonic anhydrase (4.2.1.1)	8
Succinic dehydrogenase (1.3.99.1)	7
Glucose oxidase (1.1.3.4)	6-1/2
ATPase (3.6.1.3)	6
Alkaline phosphatase (3.1.3.1)	6
Hexokinase (2.7.1.1)	6
Glutathione reductase (1.6.4.2)	5-1/2
Cytochrome oxidase (1.9.3.1)	4-1/2

Laboratory studies began with carbonic anhydrase and the other enzymes were then tested in order of point ranking as time permitted. Carboxylesterase was subsequently included for study.

ORGANIZATION OF LABORATORY STUDIES

Laboratory studies proceeded through the following three stages: (1) methods of enzyme analysis, (2) in vitro inhibition studies, and (3) CAM-instrument adaptation. When a problem was encountered during the investigation of a particular enzyme, the likelihood of its solution within a reasonable time and its anticipated effect on the final usefulness of the enzyme were evaluated. Based on these considerations, a decision was made whether to continue laboratory work with the enzyme. This decision-making process was necessary in order to ensure that preliminary data were obtained and an understanding of potential problems was developed for several enzymes within the time and effort limitations of this contract. Figure 1 illustrates the scope of the laboratory work for each enzyme studied.

Tasks	Succinic dehydrogenase	Alkaline phosphatase	Carboxylesterase	Glucose oxidase	Hexokinase	Carbonic anhydrase
Literature search	X	X	X	X	X	X
Selection of assay method	X	X	X	X	X	X
Purchase of enzyme and reagents	Enzyme not available	X	X	X	X	X
Modification of assay method for enzyme inhibition assay	—	X	X	X	X	X
Enzyme test with battery of toxic substances	—	X	—	X	X	X
Determination of sensitivity of enzyme to specific inhibitors	—	—	X	—	X	X
Investigation of alternate means to measure enzyme activity electrochemically	—	—	—	—	—	X

Figure 1. Scope of enzyme studies.

SECTION 5

METHODS OF ENZYME ANALYSIS

A literature review of assay methods was conducted for each enzyme selected for study. Although many methods were available, spectrophotometric methods of analysis were preferred, as the equipment was available and procedures were simple and readily adaptable to inhibition studies. Once a defined method had been chosen, enzymes and other reagents were ordered from commercial suppliers. As noted previously, commercial availability of enzymes was a prerequisite. Because no supplier of succinic dehydrogenase (the second enzyme chosen for study) was found, it was immediately eliminated from this work.

Published methods of enzyme analysis are often designed solely for the detection of active enzyme in assay mixtures. When used for this purpose, excess quantities of substrate may be used. However, for inhibition testing, standard concentrations of enzyme and substrate are used and the effect of given quantities of toxic materials on the activity assay are measured. The standard concentrations of enzyme and substrate are critical to the detection of inhibition. For example, excess substrate may "protect" the enzyme from inhibition when the inhibitor concentration is low in comparison to the substrate concentration.

The Worthington Enzyme Manual[4] was often used as the initial source of assay methods. These methods were modified prior to testing for inhibition by toxic chemicals in order to ensure that maximum sensitivity was obtained. Curves of enzyme activity versus substrate concentration and of enzyme activity versus enzyme concentration were generated. The reactant concentrations selected were: (1) the substrate concentration (no excess substrate) allowing maximum activity and (2) the smallest assayable concentration of enzyme.

The method of adding the test compound (water soluble and organic soluble) to the reaction mixture was specified for each screening test. When organic solvents were used, the effect of the solvent on the enzyme reaction was taken into account because organic solvents, even at less than 10% by volume, can inhibit enzyme activity. When the amount of inhibition resulting from solvent effects was small, the "enzyme control" containing the organic solvent and no inhibitor represented 100% activity. Appendix B contains the revised methods of enzyme inhibition analysis used in this work and references the original sources.

SECTION 6

ENZYME INHIBITION STUDIES

The effect of known concentrations of toxic chemicals on enzyme activity was measured in vitro. The purpose of the measurements was to screen a number of chemicals against a number of enzymes to find those enzymes that were sensitive to low concentrations of specific toxic chemicals. In this way, the expected usefulness of an enzyme for water monitoring could be anticipated before any effort was given to CAM-adaptation.

GLUCOSE OXIDASE/ALKALINE PHOSPHATASE

The results of inhibition studies of glucose oxidase and alkaline phosphatase are presented in Tables 2 and 3. With the exception of a slight inhibition of alkaline phosphatase by mercury compounds, these enzymes were not significantly (>15%) inhibited by any of the target materials. Thus, glucose oxidase and alkaline phosphatase do not appear to be useful for monitoring the toxic chemicals tested.

CARBOXYLESTERASE

The toxic arylphosphate, tri-o-cresyl phosphate (TOCP), has been receiving increased attention in environmental studies conducted by EPA.[5] It has been reported that certain carboxylesterases (E.C. 3.1.1.1, sometimes called aliesterase or tributyrinase) are sensitive to inhibition by TOCP.[6] In light of this report and because it is believed that a capability for detecting TOCP would be valuable, a comparative study of the inhibition of hog liver carboxylesterase and eel cholinesterase by TOCP was conducted. Additional toxic materials were not screened.

In studies of the sensitivities of hog liver carboxylesterase (Sigma Chemical Co. No. E-3128) and eel cholinesterase to TOCP, it was demonstrated that both enzymes were rather insensitive to TOCP, i.e., a 10^{-4} M solution of TOCP produced no more than 15% inhibition of carboxylesterase or eel cholinesterase.

CARBONIC ANHYDRASE

Table 4 presents the results of inhibition studies of carbonic anhydrase. Significant inhibition (>15%) occurred at 10^{-6} M and 10^{-5} M by cyanide-containing and mercury compounds, respectively. These toxicant levels suggest that carbonic anhydrase would detect low levels of CN^- and Hg^{++} in water.

TABLE 2. INHIBITION OF GLUCOSE OXIDASE

Chemical class	Test compound	Concentration ppm[a/]	Inhibition (%)
Organophosphate pesticides	Dichlorvos (DDVP)	24	N.S.[b/]
	Dursban®	35	N.S.
	Ethion	39	N.S.
	Naled	38	N.S.
	Parathion	26	N.S.
Chlorinated hydrocarbons	Aldrin	37	N.S.
	Chlordane	41	N.S.
	DDD	32	N.S.
	DDT	36	N.S.
	Endosulfan	41	N.S.
	Endrin	38	N.S.
	Heptachlor	37	N.S.
	Lindane	29	N.S.
	Methoxychlor	38	N.S.
	Pentachlorophenol	27	N.S.
	2,4,5-T acid	26	N.S.
	2,4,5-T butyl ester	31	N.S.
	2,4,5-Trichlorophenol	20	N.S.
Cyanide compounds	Potassium cyanide	7	N.S.
	Sodium cyanide	5	N.S.
Cadmium compounds	Cadmium chloride	23	N.S.
Mercury compounds	Mercuric acetate	32	29
	Mercuric chloride	27	29
Aryl phosphates	Phenylphosphate, disodium salt	20	N.S.
	Tri-o-cresyl phosphate	37	N.S.

a/ ppm (w/v) = μg/ml. All solutions contained the test compound at levels of 10^{-4}M. (Some solutions were hazy indicating that the solubility of the inhibitor in 10% aqueous acetonitrile was exceeded.)

b/ "N.S." means that no significant inhibition (i.e.,>15%) was detected.

TABLE 3. INHIBITION OF ALKALINE PHOSPHATASE

Chemical class	Test compound	Concentration, ppm[a/]	Inhibition (%)
Organophosphates	DDVP	24	N.S.[b/]
	Dursban[R]	35	N.S.
	Endosulfan	41	N.S.
	Ethion	39	N.S.
	Naled	38	N.S.
	Parathion	26	N.S.
Carbamates	Carbofuran	22	N.S.
	Sevin	20	N.S.
Chlorinated hydrocarbons	Aldrin	37	N.S.
	Chlordane	41	N.S.
	DDD	32	N.S.
	DDT	36	N.S.
	Heptachlor	37	N.S.
	Lindane	29	N.S.
	Methoxychlor	38	N.S.
	Pentachlorophenol	27	N.S.
	2,4,5-T acid	26	N.S.
	2,4,5-T ester	31	N.S.
	Toxaphene	41	N.S.
	2,4,5-Trichlorophenol	20	N.S.
Cyanide compounds	Potassium cyanide	7	N.S.
	Sodium cyanide	5	N.S.
Mercury compounds	Mercuric acetate	32	N.S.
	Mercuric chloride	27	N.S.
Aryl phosphates	Phenylphosphate, disodium salt	20	N.S.
	Tri-o-cresyl phosphate	37	N.S.
Others	Lead acetate	38	N.S.
	Potassium permanganate	16	N.S.
	Strontium acetate	22	N.S.

a/ All solutions contained the test compound at concentrations of 10^{-4} M. (Some solutions were hazy indicating that the solubility of the inhibitor in 10% acetonitrile was exceeded).

b/ "N.S." means that no significant inhibition (i.e., >15%) was detected.

TABLE 4. INHIBITION OF CARBONIC ANHYDRASE

Test compound	Grade purity	% Inhibition at several concentrations[a/]			
		10^{-4} M	$5x10^{-5}$ M	10^{-5} M	10^{-6} M
DDT relatives:					
DDT	Anal. std.[b/] 100%	34	29	11	—
DDT	Anal. std. 97%	28	28	5	—
Methoxychlor	Commercial 90%	40	25	55	—
Chlorophenoxy compounds:					
2,4,5,-T acids	Anal. std. 99%	+6	9	7	—
2,4,5-T butylesters	Anal. std. 100%	21	26	13	—
Aldrin-toxaphene group (toxaphene family):					
Toxaphene	Anal. std. 100%	44	39	3	—
Highly halogenated aromatics:					
Pentachlorophenol	Commercial 34%	70	72	12	—
2,4,5-Tri-chlorophenol	Anal. std. 98%	35	21	8	—
Cyanide compounds:					
Potassium cyanide	Reagent	100	—	100	46
Sodium cyanide	Reagent	100	—	79	21
Mercury compounds:					
Mercuric chloride	Reagent	100	—	55	0
Cadmium compounds:					
Cadmium chloride	Reagent	21	—	—	—

a/ Percentage depression in the activity of a standard enzyme solution.
b/ Anal. std. - Analytical standards received from EPA Pesticide and Toxic Substances Effects Laboratory, Research Triangle Park, North Carolina.

All chlorinated hydrocarbons tested, except 2,4,5-T acid, inhibited carbonic anhydrase at 5×10^{-5} M. Pentachlorophenol, toxaphene, and DDT ranked first, second, and third, respectively. At concentrations of 10^{-5} M, none of these pesticides inhibited the enzyme by 15%.

Inhibition studies with several highly toxic substances have indicated the expected utility of a monitoring device based on carbonic anhydrase. An expanded capability for detection of hazardous materials could result from the adaptation of carbonic anhydrase to the CAM instrument.

HEXOKINASE

Compounds that inhibited hexokinase by 90% in vitro at 3×10^{-4} M included DDT, mercuric salts, chlordane, lindane, methoxychor, and toxaphene. As seen in Table 5, 50% inhibition was caused by exposure to aldrin and to endosulfan at concentrations of 3×10^{-4} M and to tri-o-cresyl phosphate at a concentration of 10^{-4} M.

During tests with hexokinase, an increase in sensitivity was achieved by adding the substrate, glucose, after a 5-minute incubation of hexokinase and test compound. Additional manipulations might further increase the sensitivity of this enzyme to toxic chemicals.

Adaptation of hexokinase to an automatic monitoring device is promising but was not pursued during these studies.

TABLE 5. INHIBITION OF HEXOKINASE

Test compound	% Inhibition at several concentrations[a/]		
	3×10^{-4} M	10^{-4} M	10^{-5} M
Chlorinated hydrocarbons:			
Aldrin	55	17	—
Chlordane	92	22,60[b/]	—
DDD	0	—	—
DDT	93	40	61[b/]
Endosulfan	54	59	
Heptachlor	—	40	40[b/,c/]
Lindane	100	49	55[b/]
Methoxychlor	100	26	—
Pentachlorophenol	0	—	
Toxaphene	100	52	100[b/,c/]
Trichlorophenol	26	5,68[b/]	—
2,4,5-T acid	24	—	—
Organophosphates:			
DDVP	23	—	—
Dursban	17	—	—
Naled	22	—	—
Parathion	—	12	—
Carbamates:			
Furadan	15	—	—
Inorganic Ions:			
Strontium	0	—	—
Cadmium	—	38	—
Cyanide	—	6	—
Mercury	95	77	63[b/]
Aryl Phosphates:			
Phenlyphosphate, disodium salt	13	—	—
Tri-o-cresyl phosphate	50-90	34	—
Tricresylphosphate (mixed cresols)	—	29	—

a/ Percent inhibition was calculated by assigning 100% activity to a dioxane control containing no test compound.

b/ Value was obtained using a modification of the test procedure given in this report. Glucose is added after inhibitor and enzyme are incubated for 5 min. This procedure increases the sensitivity to inhibition.

c/ Concentration was 2×10^{-5} M.

SECTION 7

CAM INSTRUMENT ADAPTATION

The goal of instrument adaptation experiments was to discover a way to incorporate alternate enzymes into an existing automatic monitoring device. This investigation was primarily restricted to methods that could make use of the electrochemical detection system of the present CAM-type instruments. Use of this approach required the selection of substrates that are catalyzed to produce sulfhydryl groups (e.g., thiocholine esters are hydrolyzed by cholinesterase to yield sulfhydryl groups, which are detected by CAM). Non-electrochemical cell methods for generating electrical signals (pH) indicative of enzyme activity were also considered. Carbonic anhydrase was the only enzyme for which these adaptation experiments were performed.

CARBONIC ANHYDRASE: ADAPTATION TO CAM

Three reactions catalyzed by carbonic anhydrase were studied for potential use in an automatic monitor. These reactions are illustrated in Figure 2.

Hydrolysis of p-Nitrophenyl Acetate

Hydrolysis of p-nitrophenyl acetate (p-NPA), Figure 2a, is the basis for the spectrophotometric assay of carbonic anhydrase activity (Appendix B-1) used in the inhibition studies. The ability to distinguish p-NPA from its enzyme-catalyzed products is a requirement for the successful use of the hydrolysis reaction of p-NPA in CAM.

A series of electrochemical studies was conducted in beakers with platinum-wire electrodes similar to those used in the CAM's electrochemical cell (Figure 3). These studies were designed to determine the electrical properties of substrate and of substrate-enzyme solutions under various electrical conditions. Voltages between platinum electrodes in a test solution were measured while the resistance, in series with the battery and electrodes, was decreased stepwise. Test liquids were a p-NPA solution (4×10^{-3} M in Tris buffer/10% acetonitrile,pH 7.4) and a p-NPA solution also containing 2×10^{-7} M bovine carbonic anhydrase (BCA). The results of the studies demonstrated that for the resistances between 10 megohms and 15000 ohms: (1) high voltage values (0.8 to 2.0 v) were obtained and (2) electrochemical cell voltages for p-NPA solutions with and without BCA were indistinguishable. The following explanations for these results are proposed: (1) each solution

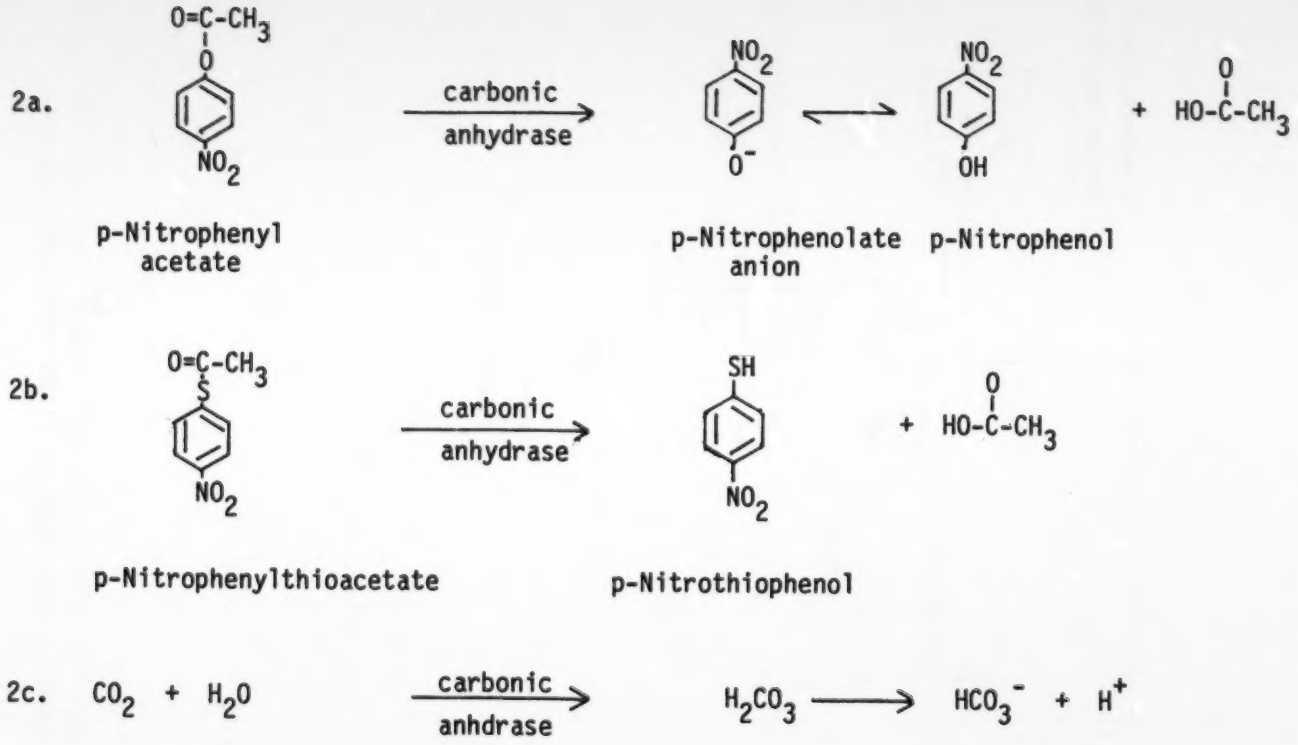

Figure 2. Carbonic anhydrase-catalyzed reactions investigated for potential use in monitoring devices.

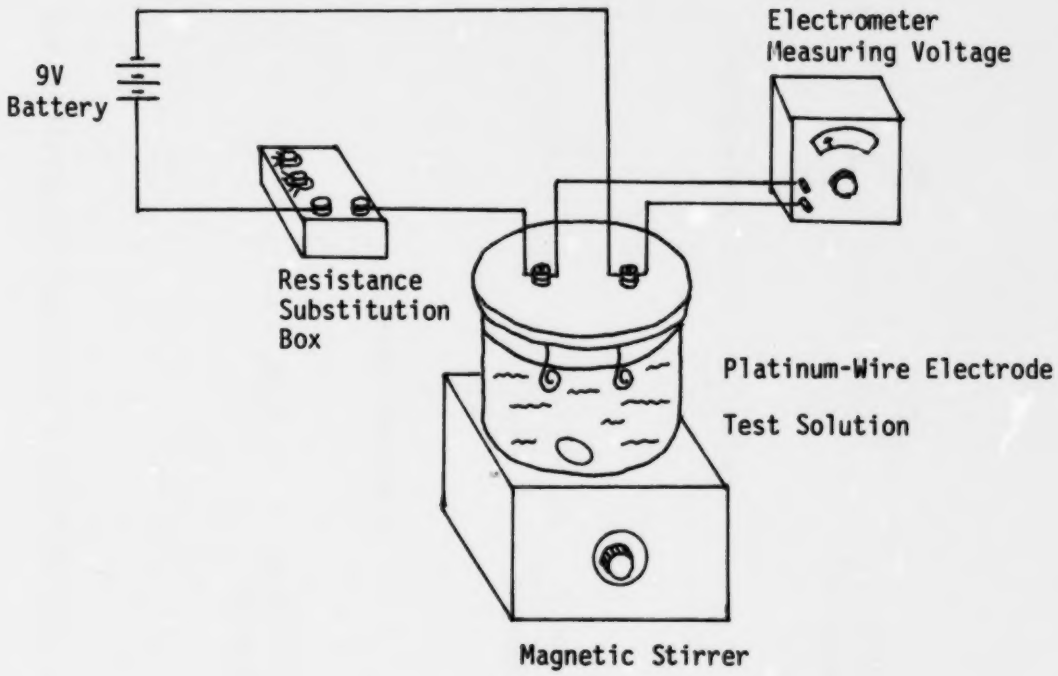

Figure 3. Apparatus for electrochemical "beaker studies". Cell potential measurements were made at fixed resistance values.

behaves only as substrate because of insufficient product formation, (2) each solution behaves only as a product because of spontaneous hydrolysis of the substrate, and (3) the substrate, p-NPA, and the reaction product, p-nitrophenol, cannot be distinguished electrochemically under the experimental conditions used.

In "beaker studies," solutions of substrate and product were examined separately under identical conditions of temperature, buffer, and pH. Fluctuations in voltage readings resulting from electrode conditioning were taken into account by repeating tests until stable and characteristic readings were obtained at each resistance. The results indicated that p-NPA could not be distinguished from p-nitrophenol electrochemically, using platinum-wire electrodes; therefore, p-NPA was abandoned as a substrate.

Hydrolysis of p-Nitrophenylthioacetate

Theoretically, the sulfur analogue of p-NPA, i.e.,p-nitrophenylthioacetate (p-NPTA), was very promising for use with carbonic anhydrase in CAM, although the use of p-NPTA as a carbonic anhydrase substrate had not been previously reported. Because it was theorized that the hydrolysis product, p-nitrothiophenol (p-NTP), Figure 2b, would lend itself to electrochemical detection due to the presence of a free sulfhydryl group, further studies were conducted.

Enzyme-catalyzed hydrolysis of p-NPTA in solution was examined spectrophotometrically and electrochemically, as follows:

Spectrophotometric Assay--

Upon standing, solutions of p-NPTA were observed to yellow with time. The yellow color presumably indicated the formation of the hydrolysis product. Clear, colorless solutions of p-NPTA were prepared by dissolving the material in acetonitrile, which was added to cold diethylmalonate buffer (pH 7.2) and kept at 4°C. Scans (350 to 850 nm) of the clear p-NPTA solutions and of the yellow, hydrolyzed material showed that spectrophotometric observations of the reaction could be made at 410 nm.

The analysis was performed by preparing clear solutions of p-NPTA as described above. A known amount of carbonic anhydrase was placed in the sample cuvette of a Beckman Model 25 spectrophotometer. Buffered substrate was added to both reference and sample cuvettes and the OD_{410} recorded for 5 min. In this way, spontaneous hydrolysis was automatically subtracted from the sample and enzymatically-catalyzed product formation was observed.

Assays done in this manner revealed a greater activity/min in the sample cuvette in all instances. Further proof of actual carbonic anhydrase activity towards p-NPTA was that the moles of substrate hydrolyzed per minute decreased as enzyme concentration in the sample was reduced ten-fold. Detailed studies to characterize and optimize this reaction were not conducted.

Electrochemical Assay--

Cold solutions of p-NPTA and p-NTP (5×10^{-4} M) in 0.01 M diethylmalonate buffer-10% acetonitrile were prepared. Measurements obtained with platinum-wire electrodes at fixed resistance values are shown in Table 6. Lower voltages were consistently observed with solutions of p-NTP. Therefore, it appeared that the hydrolysis product produced the low voltages characteristic of sulfhydryl groups, thus allowing differentiation between substrate and product in the CAM instruments.

Hydrolysis of p-NPTA was further tested in a breadboard CAM system, which operates on the same water-substrate-current cycle as CAM and uses the same platinum electrodes. A starch pad with no enzyme was placed between the electrodes and baselines were determined with the test solutions. Water was pumped through the pad during the sample cycle. The two compounds, p-NPTA and p-NTP, were distinguishable in CAM at 5×10^{-4} M. Baselines with p-NPTA were ca. 750 mv, while p-NTP produced baselines of ca. 250 mv. In principle, this electrochemical difference should be quite satisfactory for monitoring the activity of an enzyme and its subsequent inhibition. Thus, the use of p-NPTA and carbonic anhydrase in the CAM instrument remains theoretically possible. However, there are several problems associated with adaptation of this enzyme system for use in a real-time monitoring device. The problems, yet to be resolved, are as follows:

1. p-NPTA is not very soluble in aqueous solutions. Mixed solvents must be used; however, some organic solvents inhibit enzyme activity.

2. The substrate, p-NPTA, hydrolyzes spontaneously in a 10% organic-aqueous solution at room temperature. A method for handling the p-NPTA so that it remains intact prior to enzymatic hydrolysis must be devised.

TABLE 6. EXAMINATION OF p-NITROPHENYLTHIOACETATE AND p-NITROTHIOPHENOL WITH PLATINUM-WIRE ELECTRODES

Resistance at substitution box, megohms	Current, μA	Voltage with p-NPTA, v	Voltage with p-NTP, v	Voltage difference, mv
10.0	0.87	1.1	0.31	790
6.8	1.3	1.15	0.33	820
4.7	1.8	1.2	0.35	850
3.3	2.6	1.27	0.38	890
2.2	3.9	1.37	0.44	930
1.5	5.7	1.45	0.50	950
1.0	8.4	1.52	0.59	930
0.68	12.2	1.6	0.70	900
0.47	17.3	1.68	0.85	830

3. The specific activity of carbonic anhydrase towards p-NPTA is very low. A way must be found to generate sufficient quantities of product for detection during a sampling cycle of reasonable length.

4. An immobilized carbonic anhydrase product with sufficient activity towards p-NPTA must be prepared to allow read-out in the electrochemical cell of CAM. Preliminary studies of three methods for immobilization of carbonic anhydrase were conducted: (1) starch-gel entrapment on open-pore polyurethane foam, (2) photoimmobilization on a solid support, and (3) hydrophobic adsorption to phenoxyacetyl cellulose.

Starch-gel entrapment--Carbonic anhydrase was substituted for cholinesterase (ChE) in the procedure for preparing the ChE pads presently used in CAM instruments.[7,8] The maximum weight of carbonic anhydrase allowing convenient handling was used and no attempt was made to modify the ChE procedure to better accommodate carbonic anhydrase. These carbonic anhydrase pads did not produce the low baseline voltage expected of an active enzyme pad in the CAM system.

Photoimmobilization--Photoimmobilization with stepwise bifunctional reagents (e.g., 4-azido-2-nitrophenyl-γ-aminobutyryl N-oxysuccinimide) for covalent binding of enzymes to a variety of solid supports has been successfully used.[9,10] Enzymes have been coupled to matrices such as paper, cotton, nylon cloth, and polyurethane with good retention of activity. Carbonic anhydrase was photoimmobilized on a 1 cm^3 cellulose sponge in an attempt to make a product with greater absolute activity and less enzyme wash-out. Activity on the sponge after photolysis was not assayed spectrophotometrically. The product was qualitatively evaluated by testing it in CAM. Substrate was passed through the sponge and into the electrochemical cell. Again, the low voltage characteristic of the active enzyme was not seen.

Hydrophobic adsorption--A new product from Regis Chemical Company,[11,12,13] Enzorb-A (phenoxyacetyl cellulose), is available for immobilization of enzymes and other proteins by means of hydrophobic adsorption. Immobilization can be accomplished by passing a solution of enzyme or other proteins through an Enzorb-A column. This material reportedly binds 0.3 to 0.5% of its own weight in protein, often with near 100% retention of enzyme activity. This relatively simple procedure seemed promising for the immobilization of carbonic anhydrase. It was envisioned that such an enzyme column could be placed in the continuous flow system of CAM in front of the electrochemical cell.

Enzorb-A (2 g) was carried through the batch preparation given in the technical bulletin[11] using 10 mg of carbonic anhydrase. Alphacel (Nutritional Biochemical Corporation), a cellulose product with no phenoxyacetyl groups, served as the control material. All filtrates and washes from Enzorb-A and Alphacel were analyzed for carbonic anhydrase activity spectrophotometrically. Activity unaccounted for (representing enzyme remaining on the column) was calculated by difference. The Enzorb-A retained 2.9 mg carbonic anhydrase, while 0.08 mg remained on the Alphacel control column.

Although the data indicated binding of the enzyme protein, the column was not immediately usable with the electrochemical cell as an indicator of enzyme activity. Several suggestions for modifications in column preparation and techniques for operating the column in CAM remain to be explored.

In summary, the inability to produce carbonic anhydrase products usable in CAM by starch-gel entrapment, photochemical immobilization, and hydrophobic column preparations during this report period was not unexpected. These results should also be viewed in light of the fact that carbonic anhydrase has a low affinity for the substrate, p-NPTA, usable in CAM, in contrast to the high affinity of cholinesterase for choline esters. In the cholinesterase system, an enzyme preparation containing relatively little enzyme can convert sufficient substrate to be detectable by CAM, but this is not the case with carbonic anhydrase. It is anticipated that greater quantities of active enzyme, longer substrate incubation times, or higher incubation temperatures will be required for use of carbonic anhydrase in CAM. The preliminary experiments performed under this contract have provided a preview of possible adaptation techniques and an understanding of the potential problems to be solved.

Hydrolysis of Carbon Dioxide

Figure 2c illustrates the naturally occurring, "physiological" reaction of carbonic anhydrase. The specific activity of carbonic anhydrase towards the substrate, CO_2, is much greater than that towards either p-NPA or p-NPTA. With the device shown in Figure 4, the use of CO_2 as substrate and of carbonic anhydrase as enzyme was tested. Development of this apparatus was an attempt to instrument the Wilbur-Anderson electrometric assay of carbonic anhydrase activity (Appendix B-2), which measures the time required for a saturated CO_2 solution to lower the pH of a 0.02 M Tris-HCl buffer from 8.3 to 6.3 at 0°C. It was believed that a pH measurement of buffer solution (with or without carbonic anhydrase) might produce an electrical signal indicative of the presence or absence of enzyme activity and could be usable in a continuous monitor for carbonic anhydrase inhibitors.

A series of experiments was designed to establish a set of operating parameters under which time differences in the pH drop for tests with and without carbonic anhydrase could be observed (note that the CO_2-hydrolysis reaction proceeds in the absence of enzyme). Initial tests in which CO_2 and buffer solutions entered a T-tube connector and flowed directly to a pH electrode produced very "noisy" pH readings. Incorporation of a short coiled tube between the T-tube connector and pH electrode did not correct the noise problem. However, using a mixing vessel or a large T-connector with an internal stirring bar (Figure 4), smooth recordings of pH with time were generated. The following parameters were varied while pH tracings were made:

1. Flow rates of CO_2 and pH 8.0 buffer (thereby changing ratios of the two reactants and the time interval between mixing and pH measurement).

2. Mixing vessels (thereby changing time delay prior to pH measurement).

3. Temperature (reactants were mixed at room temperature (25°C) or at 4°C).

In this series of experiments, a time difference in pH drop with and without carbonic anhydrase was not seen. The probable reason for this result was that the lag time between contact of reactants and measurement of pH was too long.

To ascertain which time intervals between mixing of reactants and pH measurements might be required in a flow-through system, the electrometric carbonic anhydrase assay method[14,4] was evaluated at room temperature and at 4°C. The results are shown in Table 7. The time required for the pH to fall from 8.3 to 6.3 was shown to be temperature dependent. At 4°C, the "time window" was 27 sec, while at 25°C, it was 6 sec. In both cases, the concentration of enzyme was 10 mg per 10 ml of a buffer/CO_2 mixture. (Notice that the windows were shortened when the enzyme concentration was reduced ten-fold).

In order to detect enzyme activity on a continuous basis, as in the CAM system, measurements must be made in the time window bracketed by the blank and test times. Therefore, careful consideration of design parameters, including a provision for refrigeration, would be required to develop a carbonic anhydrase monitor such as that tested.

In conclusion, the preceeding discussions demonstrate the feasibility of adapting a carbonic anhydrase inhibition-based system for use in the CAM instrument to monitor chlorinated hydrocarbons and cyanide-containing and mercury-containing compounds. The following systems appear most promising for the development of a carbonic anhydrase inhibition-based water monitor:

TABLE 7. RESULTS OF ELECTROMETRIC ASSAY OF CARBONIC ANHYDRASE ACTIVITY

Temperature, °C	Carbonic anhydrase in reaction, mg	Test time,[1] sec	Time,[2] sec
4	Blank	30.0	—
	0.01	3.0	27.0
	0.001	20.0	10.0
25	Blank	8.0	
	0.01	2.0	6.0
	0.001	6.0	2.0
25[3]	Blank	15.0	
	0.01	2.0	13.0

1/ Time required for pH drop from 8.3 to 6.3; average of several tests
2/ Blank time minus test time="time window"
3/ A 3-ml saturated CO_2 solution was used instead of a 4-ml solution

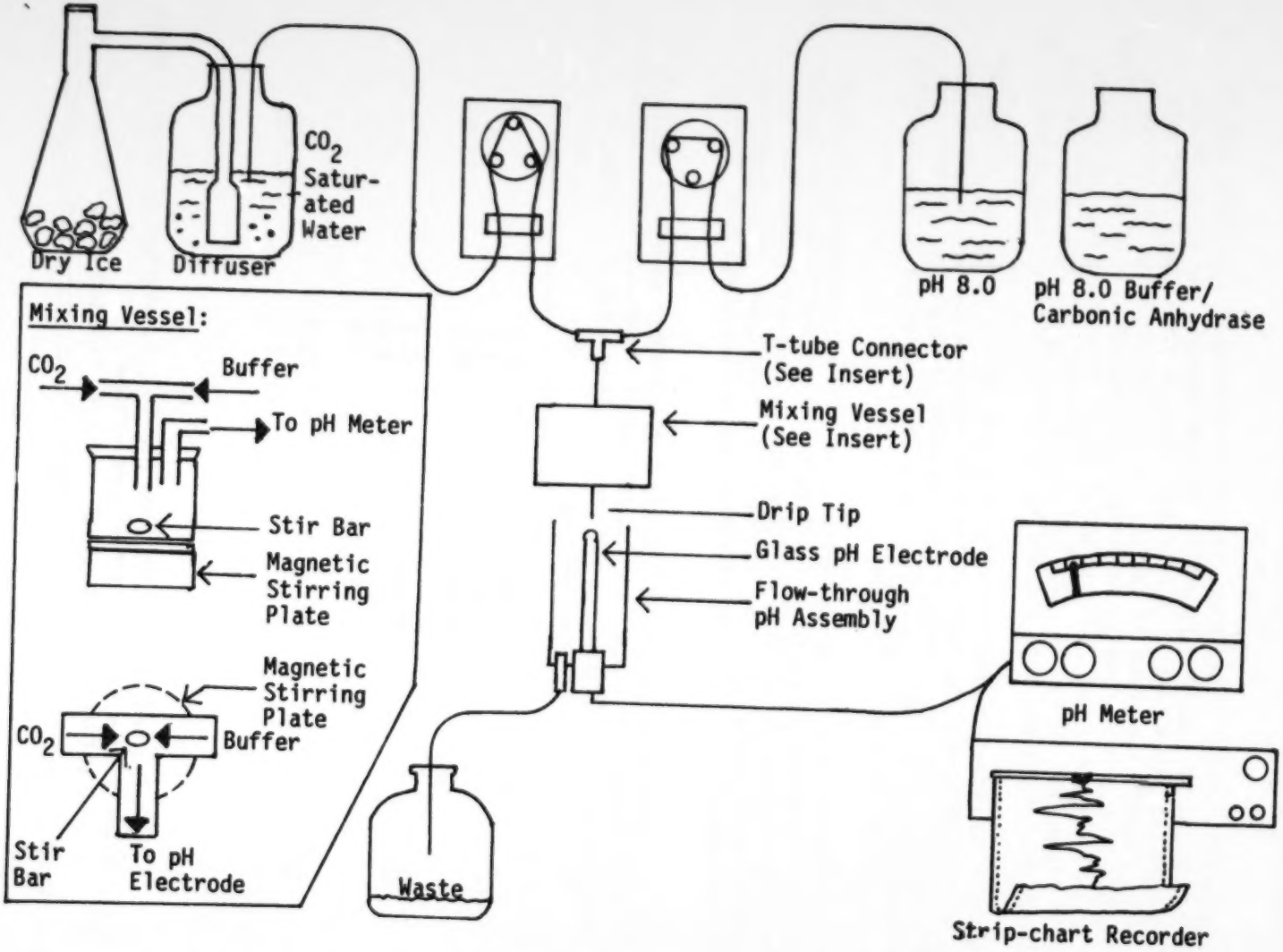

Figure 4. Prototype apparatus for determination of carbonic anhydrase activity.

1. Hydrolysis of p-nitrophenylthioacetate (p-NPTA): p-NPTA is hydrolyzed in the presence of carbonic anhydrase to form p-nitrothiophenol. As in the present CAM system--using cholinesterase--enzyme inhibition by toxic materials can be detected electrochemically by monitoring production of the -SH group in the hydrolysis product. Although the detection system would remain the same, several problems, such as low specific activity for p-NPTA hydrolysis and development of a method of enzyme immobilization, must be solved before a carbonic anhydrase /p-NPTA system could be adapted to the CAM instrument.

2. Hydrolysis of carbon dioxide (CO_2): In the presence of carbonic anhydrase, p-NPTA is rapidly hydrolyzed in solution to form hydrogen and bicarbonate ions, causing a pH change. The presence of toxic, enzyme-inhibiting materials in water will affect the residual enzyme activity, which can be determined by monitoring the pH. Utilization of a carbonic anhydrase/CO_2 system would require major changes in the detection system of the CAM instrument as presently configured. However, instrumentation of the Wilbur-Anderson assay method previously described appears to be a practicable detection alternative for use in an automatic water monitor such as CAM.

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APPENDIX A

FACT SHEET FOR CANDIDATE ENZYMES

A-1: GLUCOSE OXIDASE (1.1.3.4)

Reaction Catalyzed: Glucose + H_2O + $O_2 \longrightarrow H_2O_2$ + gluconic acid

Source: Worthington (Aspergillus niger) Cost: $45.00/100 mg (130 units/mg)

Stability: Stable 6-12 months at 4°C

Specific Activity: 80 IU/mg

Substrate Specificity: Relative rates - D-glucose=100, D-mannose=20, 2-deoxy-D-glucose=20, other hexoses=negligible

Cofactor Requirements: None

Inhibitors: Ag^+, Hg^{++}, Cu^{++}, H_2O_2, F^- (halides $F^- \gg Cl^-$, Br^-)

Electrochemical Detection: H_2O_2 + RSH$\longrightarrow$R-S-S-R

1. The formation of the reaction product, H_2O_2, could be monitored electrochemically. A sulfhydryl group could be fed into the system and, in the presence of active enzyme, H_2O_2 would oxidize the sulfhydryl compound and produce characteristic low voltages, as seen in the absence of enzymes in the cholinesterase-based system. When the enzyme is inhibited, H_2O_2 production would decrease and the lower rate of sulfhydryl oxidation would produce an increase in the cell voltage.

2. Yellow Springs Instrument Model 25 Oxidase Meter could be used for direct electrochemical detection.

3. The reaction could be coupled with glutathione peroxidase (1.11.1.9) and the loss of -SH monitored.

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Williams III, D. C., G. F. Huff, and W. R. Seitz. "Evaluation of Peroxyoxalate Chemiluminescence for Determination of Enzyme Generated Peroxide." Anal. Chem., 48(7):1003-1006 (1976).

A-2: XANTHINE OXIDASE (1.2.3.2)

Reaction Catalyzed: Xanthine + H_2O + O_2 $\longrightarrow$ urate + H_2O_2

Source: Gallard-Schlesinger (buttermilk) Cost: $40.00/100 mg (0.5 IU/mg)

Stability: Ammonium sulfate suspensions of the enzyme are stable for weeks when refrigerated and for several days at room temperature.

Specific Activity: 1.35 IU/mg

Substrate Specificity: Exhibits low specificity and attacks a number of aldehydes, purines, pteridines, pyrimidines, azopurines, and other heterocyclic compounds. Substrates: xanthine, acetaldehyde, benzaldehyde

Cofactor Requirements: None

Inhibitors: Quinazolines, benzodiarone, cyanide ion, alkylsalicylic acids

Electrochemical Detection: Detect H_2O_2 directly or monitor its reaction with RSH[2] (See Appendix A-1 Glucose Oxidase; Electrochemical Detection of H_2O_2).

References: Massey, V., et al. "On the Mechanism of Inactivation of Xanthine Oxidase by Cyanide." J. Biol. Chem., 245:6595-6598 (1970).

McCoubrey, A., et al. "Inhibition of Enzymes by Alkylsalicylic Acids." J. Pharm. Pharmacol., 22:333-337 (1970).

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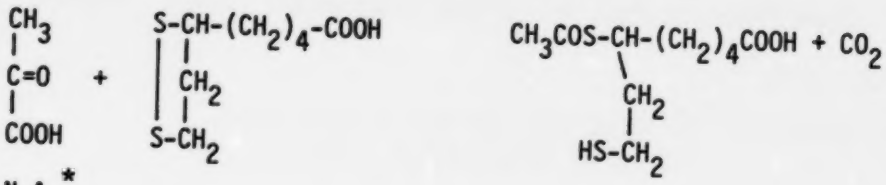
Marcolongo, R., et al. "The Inhibition of Xanthine Oxidase Activity by Benzodiarone." Clin. Chim. Acta., 41:91-94 (1972).

A-3: PYRUVATE DEHYDROGENASE (1.2.4.1)

Reaction: Pyruvate + oxidized lipoate ⟶ 6-S-acetyl-lipoate + CO_2
Catalyzed



Source: N.A.*

Stability: N.A.

Specific Activity: 0.5 IU/mg

Substrate Specificity: Will decarboxylate 2-oxobutyrate in addition to pyruvate, but not 2-oxoglutarate.

Cofactor Requirements: None, but thiamine pyrophosphate has been used.

Inhibitors: N.A.

Electrochemical Detection: Monitor the formation of -SH groups.

References: Wieland, O. H. "On the Mechanism of Irreversible Pyruvate Dehydrogenase Inactivation in Liver Mitochondrial Extracts." FEBS Letter, 52(1):44-47 (1975).

*"N.A." - Information not available.

A-4: SUCCINATE DEHYDROGENASE (1.3.99.1)

Reaction Catalyzed: Succinate + acceptor = fumarate + reduced acceptor

Source: Sigma Chemical Co., No. S-3630 (bacterial) Cost: Inquire

Stability: N.A.*

Specific Activity: 55 IU/mg (bovine heart; succinate)
5.5 IU/mg (Micrococcus lactilyticus)

Substrate Specificity: The bovine heart enzyme oxidizes succinate, L-methylsuccinate, L-ethylsuccinate, L-chlorosuccinate, and D- and L-malate.

Cofactor Requirements: None. PMS (phenazine methosulfate) is the best electron acceptor. FP (ferriflavoprotein) is another acceptor.

Inhibitors: Mirex, DDT, toxaphene, endrin, dieldrin, Guthion^{R}, manganese

Electrochemical Detection:

1. Investigate the possibility of directly monitoring the oxidation-reduction of the electron acceptor.

2. The reduced acceptor could possibly be oxidized by R-S-S-R to liberate the electroactive R-SH group.

References: Bernath, P. and T. P. Singer. "Succinic Dehydrogenase," Methods in Enzymology, Vol. 5. Academic Press, New York (1964), pp.597-614.

Davis, K. A. and Y. Hatefi. "Succinate Dehydrogenase. I. Purification, Molecular Properties and Substructure." Biochem., 10(13):2509-2516 (1971).

Hanstein, W. B., K. A. Davis, M. A. Ghalambor, and Y. Hatefi. "Succinate Dehydrogenase. II. Enzymatic Properties." Biochem., 10(13):2517-2524 (1971).

Heidker, J. C., et al. "Inhibition of Mitochondrial Electron Transport by Guthion^{R}, Some Related Insecticides and Degradative Products." Bull. Environ. Contam. Toxicol., 8:141-146 (1972).

McPhail, L. C. and C. C. Cunningham. "The Role of Protein and Lipids in Stabilizing the Activity of Bovine Heart Succinate Dehydrogenase." Biochem., 14(6):1122-1130 (1975).

*"N.A." - Information not available.

Seth, P.K., et al. "In Vitro Inhibition of Succinic Dehydrogenase by Manganese and its Reversal by Chelating Agents." Environ. Physiol. Biochem., 4(4):176-180 (1974).

Singer, T. P. "Determination of the Activity of Succinate, NADH, Choline, and Glycerophosphate Dehydrogenases." Methods of Biochem. Anal., 22:123-175 (1974).

Singer, T. P., E. B. Kearney, and W. C. Kenney. "Succinic Dehydrogenase." Advances in Enzymology, 37:189-272 (1973).

Yarbrough, J. D. and G. B. Moffett. "The Effects of DDT, Toxaphene, and Dieldrin on Succinic Dehydrogenase Activity in Insecticide-Resistant and Susceptible Gambusia Affinia." J. Agric. Food Chem., 20(3):558-560 (1972).

Yarbrough, J. D. and M. R. Wells. "Vertebrate Insecticide Resistance: The In Vitro Endrin Effect on Succinic Dehydrogenase Activity in Endrin-Resistant and Susceptible Mosquitofish." Bull. Environ. Contam. Toxicol., 6(2):171-176 (1971).

Yarbrough, J. D. and F. M. McCorkle. "The In Vitro Effects of Mirex on Succinic Dehydrogenase Activity in Gambusia Affinis and Lepomis Cyanellus." Bull. Environ. Contam. Toxicol., 11(4): 364-370 (1974).

Zanetti, G., Y. M. Galante, P. Arosio, and P. Cerletti. "Interactions of Succinate Dehydrogenase With Cyanide." Biochim. Biophys. Acta., 321:41-53 (1973).

Zeijlemaker, W. P., D. V. Dervartanian, C. Veeger, and E. C. Slater. "Studies on Succinate Dehydrogenase. IV. Kinetics of the Overall Reaction Catalyzed by Preparations of the Purified Enzyme." Biochem. Biophys. Acta., 178:213-224 (1969).

A-5: GLUTATHIONE REDUCTASE (1.6.4.2)

Reaction Catalyzed: Reduced NAD(P) + oxidized glutathione $\longrightarrow$ NAD(P) + 2 glutathione

Source: Sigma Chemical Co., No. G-4751, (yeast) Cost: $6.30/mg (100 units/mg)
Sigma Chemical Co., No. G-6004 (wheat germ) $25.00/g (100 units/g)

Stability: N.A.*

Specific Activity: Yeast=64.4, human erythrocyte=61, rat liver=839

Substrate Specificity: Specific for NAD(P)

Cofactor Requirement: Reduced NAD(P)

Inhibitors: 5-Nitro furaldehyde and nitrobenzene derivatives, mercury

Electrochemical Detection: Detection of -SH, as with cholinesterase-based system.

References: Kosmidevs, S. "Glutathione Level in the Blood in Experimental Poisoning with Metallic Mercury." Med. Pracy., 16:206-210 (1965).

Mykkanen, H.M. "Effect of Mercury on Erythrocyte Glutathione Reductase Activity." Bull. Environ. Contam. Toxicol., 12(1): 10-16 (1974).

Pekkanen, T. J. "The Effect of Experimental Methyl Mercury Poisoning on the Activity of the TPNH-Specific Glutathione Reductase of Rat Brain Liver." Acta Vet. Scand., 13:14-19 (1972).

Yawate, Y., et al. "Red Cell Glutathione Reductase: Mechanism of Action of Inhibitors." Biochem. Biophys. Acta, 321:72-83 (1973).

*"N.A." - Information not available.

A-6: THIOL OXIDASE (1.8.3.2)

Reaction Catalyzed: $4\ R{:}CR'{\cdot}SH + O_2 \longrightarrow 2\ R{:}CR'{\cdot}S{\cdot}S{\cdot}CR'{:}R + 2H_2O$

Source: Bacteria, yeast, liver, heart, muscle

Stability: N.A.*

Specific Activity: 19.8 IU/mg (thiophenol)

Substrate Specificity: The following substrates were oxidized: thiophenol, catechol, diethyldithiocarbamate, methylmercaptoimidazole, and resorcinol.

Cofactor Requirements: None

Inhibitors: Iodoacetic acid, iodoacetamide, N-ethylmaleimide, p-chloromercuribenzoate, bromoacetyl-CoA, arsenic trioxide

Electrochemical Detection:

1. Detection of -SH, as with cholinesterase-based system.
2. Use of an oxygen detector.

References: Barman, T. E. Enzyme Handbook, Vol. 1. Springer-Verlag, New York (1969), p. 220.

Boyer, P. D. (ed.). The Enzymes, 3rd Ed., Vol. 7. Academic Press, New York (1972), p. 391.

Dixon, M. and E. C. Webb. Enzymes, 2nd Ed. Academic Press, New York (1964).

*"N.A." - Information not available.

A-7: GLUTATHIONE DEHYDROGENASE (1.8.5.1)

Reaction Catalyzed: 2 Glutathione + dehydro-ascorbate $\longrightarrow$ oxidized glutathione + ascorbate

Source: N.A.*

Stability: N.A.

Specific Activity: N.A.

Substrate Specificity: Dehydro-D-araboascorbate and 1,2,3-triketocyclopropane are reduced, but at lower rates than dehydroascorbate.

Cofactor Requirements: None

Inhibitors: Unknown

Electrochemical Detection: Detection of -SH, as with present cholinesterase-based system.

References: Dixon, M. and E. C. Webb. Enzymes, 2nd Ed. Academic Press, New York (1964), p. 374.

*"N.A." - Information not available.

A-8: CYTOCHROME OXIDASE (1.9.3.1)

Reaction Catalyzed: 4 Ferrocytochrome c + O_2 = 4 Ferricytochrome c + $2H_2O$

Source: Gallard-Schlesinger (beef heart) Cost: $15/vial (25 U/vial)

Substrate Cost: $50/100 mg (Sigma from concuda)
$8.00/100 mg (Sigma from equine heart)

Stability: N.A.*

Specific Activity: 6.7 IU/mg (Pseudomonas)

Substrate Specificity: Cytochrome c from various sources. Relative rates- P. aeruginosa=100, baker's yeast=4.5, beef=0.5

Cofactor Requirements: None

Inhibitors: Cyanide, sulfide, azide, fluoride, hydroxylamine, metyrapone

Electrochemical Detection:

1. Investigate the possibility of directly monitoring the oxidation-reduction of ferrocytochrome c.

2. Use of an oxygen detector.

References: VanBuuren, K. J. "Binding of Cyanide to Cytochrome aa." Biochem. Biophys. Acta, 256:243-257 (1971).

Harmon, H. J., et al. "Inhibition of Cytochrome c Oxidase by Hydrophobic Metal Chelators." Biochim. Biophys. Acta, 368(1): 125-129 (1974).

Muijsers, A. O. "Biochemical and Biophysical Studies on Cytochrome Oxidase. IV. Reaction with Fluoride." Biochim. Biophys. Acta, 333:430-438 (1974).

Nicholls, P. "Biochemical and Biophysical Studies on Cytochrome aa 3.8. Effect of Cyanide on the Catalytic Activity." Biochim. Biophys. Acta, 275:279-287 (1972).

Nicholls, P. "Inhibition of Cytochrome c Oxidase by Sulphide." Biochem. Soc. Trans., 3(2):316-319 (1975).

Roots, I. and A. G. Hildebrandt. "Non-Competitive and Competitive Inhibition of Mixed Function Oxidase in Rat Liver Microsomes by Metyrapone." Naunyn-Schmiedeberg's, Arch. Pharmacol., 227:27-38(1973).

*"N.A." - Information not available.

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Yoshikawa, S. "The Inhibition Mechanism of the Cytochrome Oxidase Reaction IV. The Nature of the Kinetically Inactive Complex of Cytochrome Oxidase with Cyanide." J. Biochem. (Tokyo), 73:637-645 (1973).

Yoshikawa, S. "Classification of Inhibitors Based on Modes of Action." J. Biochem. (Tokyo), 71:859-872 (1972).

Yoshikawa, S. "The Inhibition Mechanism of the Cytochrome Oxidase Reaction. Inhibition by Hydroxylamine." J. Biochem. (Tokyo), 68:145-156 (1970).

A-9: CATALASE (1.11.1.6)

Reaction Catalyzed: $2H_2O_2 \longrightarrow 2H_2O + O_2$

Source: Worthington (bovine liver) Cost: $27.00/100 ml (3,000 U/ml)
Worthington (human erythrocyte) $45.00/2 ml (160,000 U/ml)

Stability: All preparations stable 6-12 months at 5°C. Do not freeze.

Specific Activity: N.A.*

Substrate Specificity: Alkyl hydrogen peroxides can replace H_2O_2 as substrate. Ethanol, formate, nitrous acid, and thiol compounds can replace the second H_2O_2 as hydrogen donor.

Cofactor Requirements: None

Inhibitors: Cu^{++}, ascorbate, sodium azide, formaldehyde, hydrazine, pyrazole

Electrochemical Detection:

1. Detection of H_2O_2 directly or indirectly. (See Appendix A-1, Glucose Oxidase, Electrochemical Detection.)

2. Use of an oxygen detector.

References: Ceniotti, G., et al. "Anti-Catalase Activity of Phenylenediamines In Vitro and In Vivo." Enzymologia, 30:290-298 (1966).

Feinstein, R. N., et al. "Effect of Compounds Related to Amino Triazole on Blood and Liver Catalase IV." ANL-7535, US AEC Argonne Nat. Lab., 96 (1968).

Feytmans, E., et al. "Effect of Pyrazole on Rat Liver Catalase." Biochem. Pharmacol., 23:1293-1305 (1968).

Thurman, R. G., et al. "Inhibition of Catalase in Perfused Rat Liver by Sodium Azide." Ann. Acad. Sci., 168:348-353 (1969).

*"N.A." - Information not available.

A-10: HEXOKINASE (2.7.1.1)

Reaction Catalyzed: ATP + D-hexose = ADP + D-hexose-6-phosphate

Source: Aldrich (yeast) Cost: $13.50/10 mg (2,500 U/10 mg)

Stability: N.A.*

Specific Activity: 800 IU/mg (glucose)

Substrate Specificity: Relative rates - D-glucose=100, D-fructose=180, D-mannose=80, D-galactose=0.2, D-glucosamine=70

Cofactor Requirements: ATP

Inhibitors: Mg^{++}, disulfides, Ca^{++}, Al^{+++}, chlorinated pesticides, diisopropylfluorophosphate

Electrochemical Detection:

1. Couple with G-6-P dehydrogenase and NAP peroxidase (1.11.1.1), measuring H_2O_2 loss.

2. Couple with G-6-P dehydrogenase and glutathione reductase, measuring -SH.

References: Barnard, E. A. "Hexokinases from Yeast." Methods in Enzymology, 42:6-20 (1975).

Bigl, V., et al. "Some Properties of Soluble and Solubilized Particle-Bound Hexokinase." J. Neurochem., 18:721-727 (1971).

Bowers, L. D., et al. "An Immobilized-Enzyme Flow-Enthalpimetric Analyzer: Application to Glucose Determination by Direct Phosphorylation Catalyzed by Hexokinase." Clin. Chem., 22(9): 1427-1433 (1975).

Britton, H. G., et al. "The Order of Addition of Substrates to Yeast Hexokinase." Biochem. J., 128:104P (1972).

Coburn, H. J. and J. J. Carrol. "Improved Manual and Automated Colorimetric Determination of Serum Glucose with Use of Hexokinase and Glucose-6-Phosphate Dehydrogenase." Clin. Chem., 19:127 (1973).

Colowick, S. P. "The Hexokinases." in Vol. IX, The Enzymes (P. D. Boyer, H. Lardy, and K. Myback, eds.). Academic Press, New York (1973), p. 1.

*"N.A." - Information not available.

DeAlaniz, M. J., et al. "Comparative Studies Regarding Different Ways of Expression of Enzymatic Activity." Enzymol., 38:85-88 (1970).

Derechin, M., Y. M. Rustum, and E. A. Barnard. "Dissociation of Yeast Hexokinase Under the Influence of Substrates." Biochem., 11:1793 (1972).

Genin, M.S., Y. F. Romanov, and V. Andreev. (Russian) "Antagonistic Nature of the Effect of Mg^{++} and Ca^{++} Ions on Hexokinase Activity." Biokhimiya, 37(4):732-735 (1972); Chem. Abstr., 77, 149037q(1972).

Domagk, G. F. "Diisopropylfluorophosphate." Physiol. Chem. 348:381-384 (1967).

Harrison, W. H., et al. "Aluminum Inhibition of Hexokinase." Lancet, 2:277 (1972).

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Jones, J. G. "Active-Site Thiol Groups of Yeast Hexokinase." Biochem J., 115:41P (1969).

Kanda, F., et al. "Competitive Inhibition of Hexokinase Isoenzymes by Mercurials." J. Biochem. (Tokyo), 79(3):543-548 (1975).

Morris, D. L., et al. "The Preparation of Nylon-Tube-Supported Hexokinase and Glucose 6-Phosphate Dehydrogenase and the Use of the Coimmobilized Enzymes in the Automated Determination of Glucose." Biochem. J., 147(3):593-603 (1975).

Murakami, K., et al. "Difference Between Hexokinase Isoenzymes in Sensitivity to Sulfhydryl Inhibitor." J. Biochem. (Tokyo), 74:321-341 (1973).

Otieno. S., et al. "Essential Thiols of Yeast Hexokinase Alkylation by a Substrate-Like Reagent." Biochem., 14(11):2403-2410 (1975).

Purich, D. L., et al. "The Hexokinases: Kinetic, Physical, and Regulatory Properties." Adv. Enzym. Related Areas of Mol. Biol., 39:249-326 (1973).

Ricard, J., et al. "The Theory of Alternative Substrates in Enzyme Kinetics and Its Application to Yeast Hexokinase." Eur. J. Biochem., 31:14-24 (1972).

Roustan, C., et al. "Yeast Hexokinase: Interaction with Substrates and Analogs Studied by Difference Spectrophotometry." Eur. J. Biochem., 44:353-358 (1974).

Sadar, M. H., et al. "A Specific Method for the Assay of Select Chlorinated Pesticides." J. Agric. Food Chem., 19:357-359 (1971).

Wright, W. R., et al. "Glucose Assay Systems: Evaluation of a Colorimetric Hexokinase Procedure." Clin. Chem., 17:1010-1015 (1971).

A-11: CARBOXYLESTERASE (3.1.1.1)*

Reaction catalyzed: $R\text{-}COO\text{-}R' + H_2O \longrightarrow R\text{-}COOH + R'OH$

Source: Sigma Chemical Co. and Boehringer Manheim Co.

Stability: N.A.**

Specific Activity: One unit will hydrolyze 1.0 μ mole per minute of ethyl butyrate to butyric acid and ethanol (pH 8.0, 25°C).

Substrate Specificity: Wide specificity for carboxylic esters; especially aliphatic carboxylic esters. p-Nitrophenyl acetate.

Cofactor requirements: None.

Inhibitors: Complete inhibition with DFP.

References:

Boursnell, J. C. and E. C. Webb. Nature, 164:875 (1949).

Dixon, M. and E. C. Webb. Enzymes, 2nd Ed. Academic Press, New York (1964), p. 218.

Huggins, C. and J. Sapides. "Chromogenic Substrates IV. Acyl Esters of p-Nitrophenol as Substrates for the Colorimetric Determination of Esterase." J. Biol. Chem., 170: 467 (1947).

*

**Synonyms: Carboxylic ester hydrolase, tributyrinase, hog liver esterase.
"N.A." - Information not available.

A-13: LIPASE (3.1.1.3)

Reaction Catalyzed: Triglyceride + H_2O = diglyceride + fatty acid ion

Source: Sigma Chemical Co., No. L-3001 (wheat germ) Cost: $4.50/g (7 U/mg)

Stability: Highly purified, homogenous preparations are extremely labile. Worthington's two preparations are stable 1 year refrigerated and dry.

Specific Activity: N.A.*

Substrate Specificity: Tri-, di- and mono-glycerides are attacked in decreasing order of activity.

Cofactor Requirements: None. May require emulsifiers for full activity. Calcium is required as an activator.

Inhibitors: Phosphate, magnesium, ATP, ascorbic acid, paraoxon, alcohols, sulfonyl halides, phenoxybenzamine, organophosphates

Electrochemical Detection: Use of a pH electrode.

References:

Jacks, T. J. "Phosphate Inhibition." J. Am. Oil Chem. Soc., 51:112-113 (1974).

Kryson, J. L. "Paraoxon Inhibition of an Insect Egg Lipase." Biochim. Biophys. Acta, 239:349-352 (1971).

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Maylie, M. F. "Action of Organophosphates and Sulfonyl Halides on Porcine Pancreatic Lipase." Biochim. Biophys. Acta, 276: 162-175 (1972).

Santhanam, K. "Studies on the Mechanism of Inhibition of Lipolysis by Phenoxybenzamine." Life Sci. (II), 10:437-442 (1971).

Tsai, S. C. "Inactivation with ATP, Magnesium and Ascorbic Acid." J. Biol. Chem., 248:5278-5281 (1973).

*"N.A." - Information not available.

A-14: ACETYLESTERASE (3.1.1.6)

Reaction Catalyzed: Acetic ester + H_2O = alcohol + acetate

Source: Hog kidney, oranges

Stability: N.A.*

Substrate Specificity: Greatest esterase activity toward esters of acetic acid. Hog kidney: n-propylacetate, n-propylchloroacetate, mono-, di-, and tri-acetin, and n-nitrophenyl-acetate. Orange: methyl acetate, mono-, di-, and tri-acetin, ethyl acetate, and acetylcholine.

Cofactor Requirements: None

Inhibitors: N.A.

Electrochemical Detection: Use a thiophenol acetate as substrate and monitor the appearance of -SH.

References: Barman, T. E. Enzyme Handbook, Vol. II. Springer-Verlag, New York (1969), p. 506.

*"N.A." - Information not available.

A-15: ALKALINE PHOSPHATASE (3.1.3.1)

Reaction Catalyzed: Orthophosphoric monoester + H_2O = alcohol + orthophosphate

Source: Aldrich (chicken intestine) Cost: \$36/g (0.9-2.2 U/mg)
Aldrich (E. coli) \$29/5 mg (20-30 U/mg)

Stability: Lyophilized preparation stable 1-2 years when stored at $5^{o}C$.

Specific Activity: 24.8 IU/mg (E.coli), 350 IU/mg (calf intestine)

Substrate Specificity: p-Nitrophenyl phosphate is often used as the substrate. Catalyzes a variety of phosphate esters including esters of primary and secondary alcohols, sugar alcohols, cyclic alcohols, and phenols

Cofactor Requirements: None

Inhibitors: Orthophosphate, L-tryptophan, L-phenylalanine, L-cysteine, urea, NAD, Mg, strontium, cyanide, Cd, Al, Be, periodate, permanganate, reference to insecticides

Electrochemical Detection: A thiophosphate ester, if a suitable substrate, may make detection of -SH possible as with the present cholinesterase-based system.

References:

Bamberger, C., J. Botbol, and R. L. Cabrini. "Inhibition of Alkaline Phosphatase by Beryllium and Aluminum." Arch. Biochem.Biophys., 123:195-200 (1968).

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Kshirsagar, S. G. "The Effect of Stable Strontium on the Alkaline Phosphatase Activity of Rat Tissues - In Vitro Studies." Biochem. Pharmacol., 24:13-20 (1975).

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Moss, D. W. "Influence of Metal Ions on the Orthophosphate Activities of Alkaline Phosphatase." Biochem. J., 11(2) (1964).

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Thorling, E. B and M. S. Niazy. "Inhibition of Leucocyte Alkaline Phosphatase by Cyanide In Vivo." Acta Med. Scand., 194:271-276 (1973).

A-16: ACID PHOSPHATASE (3.1.3.2)

Reaction Catalyzed: Orthophosphoric monoester + H_2O = alcohol + orthophosphate

Source: Aldrich (wheat germ) Cost: $16.00/g (0.15-0.3 U/mg)

Stability: Stable for years when maintained refrigerated as a dry powder.

Specific Activity: 93.3 IU/mg (E. freundei), 1,650 IU/mg (human)

Substrate Specificity: Broad esterase activity

Cofactor Requirements: None

Inhibitors: Heparin, formaldehyde, citrate, transition metals, reference to insecticides

Electrochemical Detection: A thiophosphate ester, if a suitable substrate, may make detection of -SH possible, as with the present cholinesterase-based system.

References: Naqui, S. N. "Studies on In Vivo Effect of Varying Doses of Insecticides of the Phosphomonoesterases of the Desert Locust." Folia Biol., 17:409-419 (1969).

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A-17: UREASE (3.5.1.5)

Reaction Catalyzed: Urea + H_2O = CO_2 + $2NH_3$

Source: Aldrich (jack bean meal) Cost: $15.00/mg (100 U/mg)
Aldrich (Bacillus sp.)

Stability: Purified urease is not very stable. Bacterial urease may be stabilized by impurities and is stable 6-12 months at 4°C.

Specific Activity: 2,130 IU/mg (jack bean), 2,430 IU/mg (Bacillus sp.)

Substrate Specificity: Absolute substrate specificity

Cofactor Requirements: None

Inhibitors: Heavy metal ions, ammonia, metal ions

Electrochemical Detection: Adapt an ammonia-specific electrode to CAM-1.

References: Hughes, R. B. "Inhibition of Urease by Metal Ions." Enzymologia, 36:332-334 (1969).

Toren, E. C. Jr. "Trace Determination of Metal Ion Inhibitors of the Urea-Urease System by a pH Stat Kinetic Method." Mikrochim Acta, 5:1049-1058 (1968).

A-18: ATPase (3.6.1.3)

Reaction Catalyzed: $ATP + H_2O = ADP + orthophosphate$

Source: Sigma Chemical Co. (pork brain) Cost: $8.00/5 U

Substrate Cost: ATP $40/10 g

Stability: N.A.*

Specific Activity: 114 IU/mg (beef heart with DNP), 24 IU/mg (skeletal muscle myosin)

Substrate Specificity: Relative rates - ATP=100, TTP=125, GTP=75, UTP=2.0

Cofactor Requirements: Mg^{++} required for activity

Inhibitors: Aldrin, dieldrin, DDT, Kepone®, toxaphene, polychlorinated biphenyls, Plictran®

Electrochemical Detection:

1. Couple the reaction to an easily monitored reaction such as DPN→DPNH.

2. Couple the reaction with isocitrate dehydrogenase (1.1.1.42) and glutathione reductase (1.6.4.2) and measure -SH.

3. Couple the reaction with isocitrate dehydrogenase (1.1.1.42) and carbonic anhydrase (4.2.1.1) and measure H^+.

References: Davis, P. W., et al. "Organochlorine Insecticide, Herbicide and Polychlorinated Biphenyl (PCB) Inhibition of NaK-ATPase in Rainbow Trout." Bull. Environ. Contam. Toxicol., 8:69-72 (1972).

Desaiah, D., et al. "Inhibition of Fish Brain ATPases by Aldrin-transdiol, Aldrin, Dieldrin and Photodieldrin." Biochem. Biophys. Res. Commun., 64(1):13-19 (1975).

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Desaiah, D., et al. "Toxaphene Inhibition of ATPase Activity in Catfish, Ictalurus Punctatus, Tissues." Bull. Environ. Contam. Toxicol., 13(2):238-244 (1975).

Desaiah, D., et al. "Inhibition of ATPase Activity in Channel Catfish Brain by Kepone and its Reduction Product." Bull. Environ. Contam. Toxicol., 13(2):153-158 (1975).

*"N.A." - Information not available.

Hexum, T. D. "Studies on the Reaction Catalyzed by Transport (Na, K) Adenosine Triphosphatase. I. Effects of Divalent Metals." Biochem. Pharmacol., 23(24):3441-3447 (1974).

Koch, R. B., et al. "Polychlorinated Biphenyls: Effect of Long-Term Exposure on ATPase Activity in Fish." Bull. Environ. Contam. Toxicol., 7:87-92 (1972).

Schneider, R. P. "Mechanism of Inhibition of Rat Brain ATPase by DDT " Biochem. Pharmacol., 24(9):939-946 (1975).

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A-19: CARBONIC ANHYDRASE (4.2.1.1)

Reaction Catalyzed: H_2CO_3 (or $H^+ + HCO_3^-$) = $CO_2 + H_2O$

Source: Aldrich (beef blood) Cost: $10.00/50 mg (3,000-4,000 U/mg)

Stability: Lyophilized enzyme shows no loss of activity when stored refrigerated for 1 year.

Specific Activity: N.A.*

Substrate Specificity: Hydration of aldehydes: acetaldehyde, 2-, 3-, and 4- pyridine aldehydes, CH_3CH_2CHO, $(CH_3)_2$ CHCHO. Esterase activity: p-nitrophenyl acetate and p-nitrophenyl thioacetate

Cofactor Requirements: None

Inhibitors: Methyl mercuric chloride, phenylmercuric chloride, lead nitrate, silver nitrate, dieldrin, DDT, chlordane, 2,4,5-T, cyanide, azide, heavy metal ions, cyanomercurials

Electrochemical Detection: Possibility of detection of -SH if esterase activity can be directed to a thioester.

References: Christenson, G. M. "Effects of Selected Water Toxicants on the In Vitro Activity of Fish Carbonic Anhydrase." Chem. Biol. Interact., 13(2):181-192 (1976).

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Maren, T. H. "Inhibition by Anions of Human Red Cell Carbonic Anhydrase B: Physiological and Biochemical Applications." Science, 191(4226):469-472 (1976).

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*"N.A." - Information not available.

Pocker, Y. and J. E. Meany. "The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. I. Kinetic Studies of the Enzyme-Catalyzed Hydration of Acetaldehyde." Biochemistry, 4(11):2535-2540 (1965).

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Wilbur, K. M. and N. G. Anderson. "Electrometric and Colorimetric Determination of Carbonic Anhydrase." J. Biol. Chem., 176:147 (1948).

A-20: S-ALKYL CYSTEINE LYASE (4.4.1.6)

Reaction Catalyzed: S-methyl-L-Cysteine = pyruvate + NH_3 + methyl mercaptan

Source: Pseudomonas cruciviae

Stability: N.A.*

Specific Activity: Variously substituted cysteines and cysteine sulfoxides

Cofactor Requirements: None

Inhibitors: N.A.

Electrochemical Detection: Detection of -SH, as with cholinesterase-based system.

References: Barman, T. E. Enzyme Handbook, Vol. II. Springer-Verlag, New York (1969) p. 812.

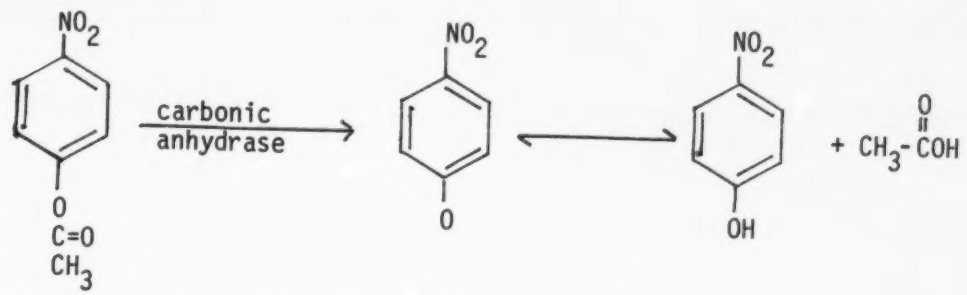
*"N.A." - Information not available.

APPENDIX B

METHODS OF ENZYME INHIBITION ANALYSIS

B-1 CARBONIC ANHYDRASE INHIBITION ASSAY METHOD*

Reaction Catalyzed



p-nitrophenylacetate | p-nitrophenolate anion | p-nitrophenol | acetic acid

Reagents

Carbonic anhydrase (4.2.1.1): BCA (bovine erythrocyte) Sigma Chemical Co. No. C-7500, Lot No. 86C-8125. 0.15 mg/50 ml buffer.

p-Nitrophenylacetate (NPA): Sigma Chemical Co. No. N-9126, Lot. No. 44C-1530.

For organic inhibitors: 4×10^{-1} M NPA (366 mg/5 ml acetonitrile)
For inorganic inhibitors: 4×10^{-2} M NPA (36.6 mg/5 ml acetonitrile)

Tris: 0.009 M, brought to a final ionic strength of 0.09 with NaCl and adjusted to pH 7.5 with concentrated HCl

*Pocker, Y. and J. T. Stone. "The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. III. Kinetic Studies of the Enzyme-Catalyzed Hydrolysis of p-Nitrophenol Acetate." Biochemistry, 6(3):668-678 (1967).

Reaction Conditions (in cuvette)

BCA: 10^{-7} M

p-NPA: 2×10^{-3} M

Acetonitrile: 10%

Buffer: 0.01 M; pH 7.2, diethylmalonate

Procedure

Inorganic Inhibitors--

1. Place 2.7 ml buffered enzyme in the cuvette.
2. Add 0.03 ml aqueous inhibitor and mix.
3. Layer 0.3 ml 0.04 M p-NPA in acetonitrile on top of the aqueous phase and mix.
4. Read the OD_{400} for 2 min.

Organic Inhibitors--

1. Place 2.7 ml buffered enzyme in the cuvette.
2. Layer 0.3 ml acetonitrile solution of inhibitor on top of the aqueous phase.
3. To this add 0.03 ml 0.4 M p-NPA standard and mix.
4. Read the OD_{400} for 2 min.

B-2 CARBONIC ANHYDRASE ASSAY, ELECTROMETRIC METHOD*

Reaction Catalyzed

$$CO_2 + H_2O \longrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$

Reagents

Lyophilized enzyme powder: Sigma Chemical Co. No. C-7500, dissolve in ice cold water (0.1 mg/ml). Store in ice bath. Immediately prior to use, dilute to a concentration of approximately 0.01 mg/ml in ice cold water.

Carbon dioxide-saturated water: Bubble CO_2 gas through 200 ml ice cold water for 30 min prior to assay. During saturation process, store water at 0^o to 4^oC in an ice bath.

Tris-HCl buffer: 0.02 M, pH 8.0. Store in an ice bath at 0^o to 4^oC before and during use.

Procedure

Blank determination--

1. Add 6.0 ml of chilled 0.02 M Tris-HCl buffer (pH 8.0) to a 10-ml beaker.
2. Maintain temperature at 0^o to 4^oC and record pH.
3. Withdraw 4 ml of chilled CO_2-saturated water and add to Tris buffer.
4. Immediately start a stop watch and record the time (T_o) required for the pH to drop from 8.3 to 6.3.

Enzyme Determination--

1. Add 6.0 ml of chilled 0.02 M Tris-HCl buffer (pH 8.0) to a 10-ml beaker.
2. Maintain temperature at 0^o to 4^oC and record pH.
3. Add 0.1 ml of freshly diluted enzyme.
4. Quickly add 4 ml of CO_2-saturated water and record the time (T) required for the pH to drop from 8.3 to 6.3.

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*Wilbur, K. M. and N. G. Anderson. "Electrometric and Colorimetric Determination of Carbonic Anhydrase." J.Biol. Chem., 176, 147 (1948).

Decker, L. A. (Ed.) Worthington Enzyme Manual. Worthington Biochemical Corporation, Freehold, New Jersey (1977), pp. 280-281.

Calculation

$$\text{Units/mg} = \frac{2(T_o - T)}{(T)\ (\text{mg enzyme in reaction mixture})}$$

T_o = time without enzyme (blank)

T = time with enzyme (test)

B-3 GLUCOSE OXIDASE INHIBITION ASSAY METHOD*

Reaction Catalyzed

$$\text{D-glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{D-glucono-S-lactone} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} \text{oxidized o-dianisidine (460 nm)}$$

Reagents

Glucose oxidase (1.1.3.4): Type V(*Aspergillus Niger*), Sigma Chemical Co. Lot No. 17C-02141. 5mg/ml (1385 units/ml). For a standard solution, make a 1:40 dilution of the enzyme, 0.125 mg/ml buffer.

18% Glucose: Allow mutarotation to come to equilibrium by standing overnight.

Potassium phosphate buffer: 0.1 M (pH 6.0)

Peroxidase: Dissolve peroxidase at a concentration of 200 µg/ml in glass distilled water.

1% o-Dianisidine: Note o-dianisidine has been reported to be carcinogenic in the solid form.

Dianisidine-buffer mixture: Prepare by dissolving 0.1 ml of 1% o-dianisidine in 12 ml of 0.1 M potassium phosphate buffer (pH 6.0).

Procedure

1. Add 0.990 ml of inhibitor solution (in 0.1 M phosphate buffer, pH 6.0/ 10% acetonitrile) to a test tube.

2. Add 0.010 ml of standard enzyme solution and incubate at room temperature for 4 min.

3. Set the spectrophotometer at 460 nm and 25°C. Pipette into cuvette as follows:

	Sample	Reference
Dianisidine-buffer mixture (pH 6.0)	2.5 ml	2.6 ml
18% Glucose	0.3 ml	0.3 ml
Peroxidase	0.1 ml	0.1 ml
Test solution of glucose oxidase inhibitor	0.1 ml	—

*Decker, L. A. *Worthington Enzyme Manual*. Worthington Biochemical Corporation, Freehold, New Jersey (1977), pp. 37-38.

4. Read the OD_{460} at room temperature for 4 min.

Calculation

$$\text{Units/mg} = \frac{\triangle OD_{460}/\text{min}}{(11.3)\ (\text{mg enzyme/ml reaction mixture})}$$

B-4 ALKALINE PHOSPHATASE INHIBIION ASSAY METHOD*

Reaction Catalyzed

p-nitrophenylphosphate $\longrightarrow$ p-nitrophenol + H_3PO_4
(410 nm)

Reagents

Alkaline phosphatase (3.1.3.1): Type III-S (Escherichia coli), Sigma Chemical Co. No. P-4377, Lot No. 32C-6030 (144 units/ml). For a standard solution, dilute to 3.7 l enzyme/250 ml buffer.

p-Nitrophenylphosphate (p-NPP): 0.05 M

Tris-HCl: 1.5 M (pH 8.0)

Reaction Conditions (in the cuvette)

Alkaline phosphatase: 2×10^{-9} M

p-NPP: 0.5 mM

Inhibitor: 10^{-4} M

Acetonitrile: 10%

Procedure

1. Set the Beckman 25 recording spectrophotometer at 410 nm.

2. Add, as follows, to the sample and reference cuvettes:

	Sample	Reference
Buffer	—	2.7 ml
Buffered enzyme	2.7 ml	—
10^{-3} M inhibitor in acetonitrile	0.3 ml	0.3 ml

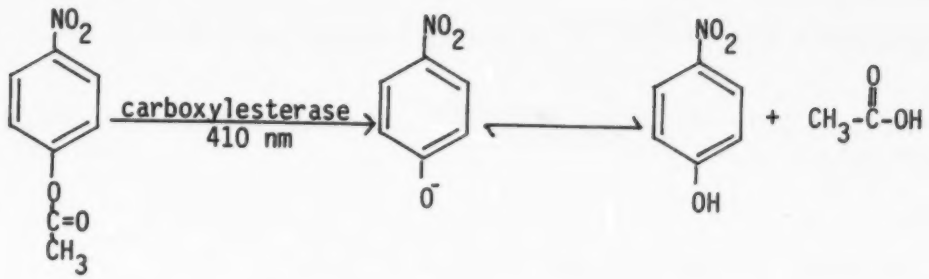
3. Mix the contents and incubate 2 min at room temperature.

4. Add 0.03 ml p-NTP to each cuvette and record the OD_{410} for 2 min.

*Decker, A. L. (Ed.) Worthington Enzyme Manual. Worthington Biochemical Corporation, Freehold, New Jersey (1977), pp. 138-140.

B-5 CARBOXYLESTERASE INHIBITION ASSAY METHOD*

Reaction Catalyzed



p-nitrophenylacetate | p-nitrophenolate anion | p-nitrophenol | acetic acid

Reagents

Carboxylesterase (3.1.1.1): Type I (porcine liver), Sigma Chemical Co. NO. E-3128, Lot No. 106C-8010 (0.065 units/ml).

p-Nitrophenylacetate: 3.5×10^{-4}

Tris: 0.02 (pH 8.0)

Procedure

1. Set the spectrophotometer at 410 nm.
2. To an aliquot of standard enzyme, add a small volume of methanolic TOCP (calculated to result in the desired test concentration).
3. Incubate at room temperature.
4. Add 3.0 ml of buffered p-NPA to the sample and reference cuvettes.
5. Add 50 µl of the esterase - TOCP (from Step 2) to the sample cuvette.
6. Record the OD_{410} for 3 min.

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*Decker, L. A. Worthington Enzyme Manual. Worthington Biochemical Corporation, Freehold, New Jersey (1977), pp. 280-281.

Sadar, M. H. and G. G. Guilbault. "A Specific Method for the Assay of Select Chlorinated Pesticides" J. Agr. Food Chem., 19(2):357359.

B-6 HEXOKINASE INHIBITION ASSAY METHOD*

Reaction Catalyzed

D-glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

glucose-6-phosphate + NAD $\xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}}$ gluconate-6-phosphate + NADH (340 nm)

Reagents

Hexokinase: 0.0146 units/ml

Glucose: 1.86 mM (in buffer)

Tris-HCl: 0.5 M (pH 8.0), with 13.3 mM/$MgCl_2$

ADP: 16.5 mM (in buffer)

NAD: 6.8 mM (in buffer)

Glucose-6-phosphate dehydrogenase: 0.97 units/ml

Procedure

1. Set the Beckman 25 recording spectrophotometer at 340 nm (UV source).
2. Add, as follows, to the sample and reference cuvetts and mix:

	Sample	Reference
Tris-$MgCl_2$ buffer	2.28 ml	2.28 ml
Glucose	0.50 ml	0.50 ml
ATP	0.10 ml	0.10 ml
NAD	0.10 ml	0.10 ml

3. Add and mix either a or b:

	Sample	Reference
a. Organic inhibitor in dioxane	0.1 ml	0.1 ml
b. Inorganic inhibitor/buffer	0.1 ml	0.1 ml

4. Add and mix:

	Sample	Reference
G-6-PDase	0.02 ml	0
Hexokinase	0.02 ml	0

5. Read at OD_{340} for 3 min.

APPENDIX C

ABSTRACTS OF PREVIOUS CAM-INSTRUMENT REPORTS

C-1 CAM-4, A PORTABLE DEVICE FOR ORGANOPHOSPHATE HAZARDOUS MATERIAL SPILLS (ENVIRONMENTAL PROTECTION TECHNOLOGY SERIES: FINAL REPORT ON TASK II, CONTRACT NO. 68-03-0299)

Previously, an instrument designated as CAM-1 ("CAM" is an acronym for "cholinesterase antagonist monitor") was constructed to continuously monitor the levels of organophosphate and carbamate pesticides in water (ponds, streams, plant outfalls, etc). CAM-1 is a sophisticated research instrument that cannot be conveniently used in the field or from a boat because of its non-ruggedized construction. To meet the requirements for an equally sensitive but portable system, CAM-4 was developed. CAM-4 is a field version of CAM-1 that will operate continuously in the field from a 12-v DC power supply for eight hours, or from 110-v AC. The present report describes the design, fabrication, and evaluation of CAM-4.

Operation of the CAM instruments is based on inactivation of the enzyme cholinesterase by organophosphate and carbamate pesticides. The extent of inactivation, which is proportional to the amount of inhibitor present, is determined by measuring the response of the system to a substrate readily hydrolyzed by the enzyme.

The conventional method for detecting organophosphates and carbamates in water requires the addition of cholinesterase, a buffer, and an enzyme-hydrolyzable substrate, e.g., butyrylthiocholine iodide, to a water sample, followed by spectrophotometric determination of the residual substrate concentration. The spectrophotomeric method is disadvantageous because: (a) a significant quantity of costly enzyme is needed for each test, and (b) the system is not easily adpated to continuous use since the optical windows become dirtied by the flow of contaminated water. It is necessary to correct this problem by comparing the response of the contaminated sample, plus reagents, to that obtained with a contaminated sample alone. However, even with this correction, frequent cleaning is necessary. The CAM systems eliminate these problems. Entrapping a given amount of enzyme in starch gel on the surface of open-pore polyurethane foam makes repeated use possible over extended periods of time. Residual activity of the enzyme is measured electrochemically.

In the CAM system, residual activity is determined during a sampling cycle by the level of substrate hydrolysis product present in the electrochemical cell. Presence of a cholinesterase inhibitor reduces the rate of

substrate hydrolysis (thiol formation) and produces an increase in cell voltage. The magnitude of the increase above the enzyme pad potential is a function of the residual enzyme activity; that is, the voltage rises as the enzyme is inhibited. A voltage increase from one sampling cycle to the next, above a designated alarm threshold, is used to trigger an "alarm" indicating the presence of cholinesterase inhibitors.

Development of a portable system necessitated elimination of the following convenience features from CAM-1: the computer logic circuits, automatic pad changer, digital voltmeter, strip chart recorder, and audible alarm components. CAM-4 contains an inverter to transform DC to AC current and a digital printer to record cell voltage. An operator must read the digital printout to determine when there is an "alarm" condition and when it is necessary to replace the enzyme pad.

CAM-4 possesses detection and monitoring capabilities equal to those of CAM-1. The sensitivity of CAM-4 to subtoxic levels of DDVP, Systox®, Furadan®, malathion, Sevin®, and other pesticides in water is comparable to the sensitivity of CAM-1 for the same pesticide solutions.

This report was submitted in fulfillment of Contract No. 68-03-0299, Task No. 2, by Midwest Research Institute under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from 3 April 1975 to 3 May 1976 and work was completed as of 3 July 1976.

C-2 EVALUATION OF "CAM-1", A WARNING DEVICE FOR ORGANOPHOSPHATE HAZARDOUS MATERIAL SPILLS (ENVIRONMENTAL PROTECTION TECHNOLOGY SERIES EPA-600/77-219, NOVEMBER 1977; FINAL REPORT ON TASK I, CONTRACT NO. 68-03-0299)

The Cholinesterase Antagonist Monitor (CAM-1) has been operated with water containing a variety of pollutants including organophosphates, carbamates, chlorinated hydrocarbons, and various other economic poisons, and its sensitivity to many of these materials has been measured. With few exceptions only the organophosphates and carbamates are detectable with CAM-1. One of these exceptions is zinc, at 10 ppm, which inactivates cholinesterase and behaves in CAM-1 like the organophosphates. Another compound detectable under certain conditions is the reversible cholinesterase antagonist, tributyl amine hydrochloride; it is detectable for only one or possibly two cycles when a sudden increase in the concentration of the reversible inhibitor occurs. The non-reversible enzyme inhibitors, on the other hand, produce repeated voltage increases until the enzyme in CAM-1 is completely inactivated. CAM-1 is recommended only for the detection of non-reversible inhibitors.

Correlation of the sensitivity of CAM-1 with the chemical structures of a group of organophosphate pesticides has shown that CAM-1 is generally more sensitive for the phosphate ($O-\overset{O}{\overset{|}{P}}=O$) compounds than for the phosphorothioate ($O-\overset{O}{\overset{|}{P}}=S$) or the phosphorodithioate ($S-\overset{O}{\overset{|}{P}}=S$) compounds, even though the animal toxicities of these different types of compounds may be very close.

Operation of CAM-1 in simulated sea water (3% NaCl) changes the voltages registered on the digital voltmeter, but it does not change the sensitivity of CAM-1 for compounds like DDVP; thus, CAM-1 is suitable for the detection of cholinesterase inhibitors in either sea or brackish waters. CAM-1 has much promise for monitoring of water supplies and plant effluents for cholinesterase inhibitors, but it is so new that it should be investigated under the conditions of intended usage prior to putting it into regular service.

C-3 RAPID DETECTION SYSTEM FOR ORGANOPHOSPHATES AND CARBAMATE INSECTICIDES IN WATER (ENVIRONMENTAL PROTECTION TECHNOLOGY SERIES EPA-R2-010, AUGUST 1972; FINAL REPORT, CONTRACT NO. 68-01-0038)

An apparatus for the detection and monitoring of water supplies for hazardous spills of organophosphate and carbamate insecticides has now been designed and fabricated. The new unit is called the Cholinesterase Antagonist Monitor, CAM-1, because it produces an alarm in 3 min when toxic or subtoxic levels of cholinesterase antagonists are present in water. Response of this apparatus to subtoxic levels of azodrin, Sevin®, dimetilan, malathion, parathion, and DDVP has already been demonstrated. CAM-1 uses immobilized cholinesterase for the collection of cholinesterase inhibitors from the water supplies. The activity of the immobilized cholinesterase is determined automatically in an electrochemical cell by passing a substrate solution over the enzyme at regular time periods. A minicomputer is used to automate the detection process and to signal an alarm when there is a rapid loss of enzyme activity--a situation that occurs in the presence of organophosphate and carbamate insecticides in the water sampled.

This report was submitted in fulfillment of Project No. 15090-GLU, Contract No. 68-01-0038, under sponsorship of the Water Quality Office, Environmental Protection Agency.